HUMAN PERIODONTAL CELLS DEMONSTRATE OSTEOBLAST-LIKE AND FIBROBLAST-LIKE CHARACTERISTICS IN TISSUE CULTURE

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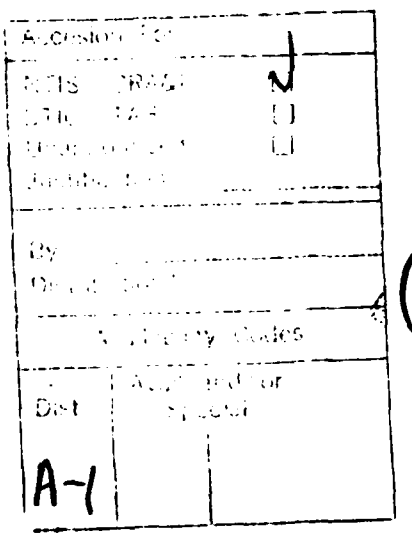

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HUMAN PERIODONTAL CELLS DEMONSTRATE OSTEOBLAST-LIKE
AND FIBROBLAST-LIKE CHARACTERISTICS
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Clinical research suggests that subpopulations of cells from the periodontium determine the mode of healing after periodontal surgery. However, few studies characterizing cells from the periodontium have been completed. Therefore the phenotypes of cells in the periodontium remain in question. The purpose of this study was to characterize the phenotypes of cells cultured from human periodontium in order to provide insight into healing mechanisms. Periodontal ligament cells were cultured from tissue scraped from third molars

(9 patients, mean age: 21 years) and gingival cells were cultured from tissue removed during periodontal surgery (6 patients, mean age: 48 years). Cell populations were characterized in the second through fifth passages for alkaline phosphatase activity, osteocalcin release, and mineralization, each after stimulation of 0 to 100 nanomolar 1,25-$(OH)_2$-vitamin D_3. Alkaline phosphatase activity was assessed on cell lysates. Osteocalcin released into the culture media of confluent cultures was assayed with a radioimmunoassay after 48 hours of 1,25-$(OH)_2$-vitamin D_3 stimulation. Mineralization was assessed with von Kossa stain after cells were cultured 2 to 4 weeks in media containing beta-glycerophosphate. Selected mineralized cultures were viewed with scanning and transmission electron microscopy.

Populations of cells with high alkaline phosphatase activity, significant osteocalcin release, and mineralization in culture, each modulated by 1,25-$(OH)_2$-vitamin D_3, were isolated from human periodontal ligament (3), gingiva (1) and selected subcultures of primary gingival explants (1). These cells were considered osteoblast-like. Fibroblast-like cell populations without significant alkaline phosphatase activity, osteocalcin release, or mineralization were isolated from the periodontal ligament (3), gingiva (2) and selected subcultures of primary gingival explants (1). Distinct cell populations in the periodontal ligament are potentially capable of regenerating the distinct components of the attachment apparatus. The presence of similar osteoblast-like cells in gingiva and periodontal ligament emphasizes similarities between the connective tissues of gingiva and periodontal ligament. Possible regeneration of periodontal attachment by cells from the attached gingiva under certain conditions is not ruled out.

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I. INTRODUCTION AND LITERATURE REVIEW

A. Regeneration

1. Regenerative periodontal therapy. Ideally periodontal therapy regenerates the periodontal attachment apparatus including alveolar bone, functional periodontal ligament fibers, and cementum. Although therapy usually ameliorates destructive periodontitis and retards disease progression (Lindhe, 1983), periodontal therapy rarely reverses general attachment loss completely (Egelberg, 1987). Under the favorable conditions in two and three walled osseous defects, surgical debridement followed by meticulous postsurgical plaque control results in partial to complete osseous repair based on radiographs, probings and reentry procedures (Patur and Glickman, 1962, Rosling et al., 1976, Polson and Heijl, 1978). Histologically, only a small portion of the defect near viable periodontal ligament regenerates in surgically debrided defects, and a long epithelial attachment replaces lost connective tissue attachment (Stahl et al., 1983).

Attempts to improve regeneration generally follow two conceptual approaches: bone morphogenic protein and guided tissue regeneration. Historically, bone morphogenic protein guided perceptions of bone regeneration using osseous grafts (Urist, 1965). Clinicians and researchers gradually realized the importance of periodontal ligament granulation tissue in periodontal regeneration (Melcher, 1976, Andreasen, 1981). Nyman et al. (1982b) demonstrated dramatic regeneration of a human periodontal defect using membranes to guide periodontal ligament tissue into the defect while excluding epithelium, gingival connective tissue, and bone.

2. Bone morphogenic protein. Bone morphogenic protein (BMP) is an acid resistant glycoprotein (or group of glycoproteins) present in low con-

centrations in bone. BMP conveys osteoinductive properties to bone matrices such as freeze dried demineralized bone. Purified BMP implanted in skeletal muscle tissue induces bone formation (Urist, 1965). Osteoblasts and chondroblasts appear to share a common mesenchymal stem cell with muscles cells, fibroblasts, and adipocytes (Owen, 1987). Under the influence of BMP mesenchymal stem cells aggregate, proliferate and differentiate into chondrocytes and osteoblasts (Figure 1A). Osteogenesis and new bone formation follow (Urist et al., 1984).

Demineralized dentin also induces bone formation in intermuscular implants (Urist, 1980). Register (1973) demineralized root dentin to stimulate bone and cementum formation. Citric acid demineralized teeth produce significant regeneration in beagle dogs (Crigger et al., 1978), but little regeneration occurs in humans (Cole et al., 1980).

Purified bone morphogenic protein promotes healing in nonunion fractures (Johnson et al., 1988). Osseous grafts also contain growth factors such as human skeletal growth factor, a high molecular weight protein (83,000 daltons) which stimulates osteoblast proliferation (Harakas, 1984).

Bone grafts improve regeneration in periodontal defects (Bowers et al., 1982). However, bone grafts usually yield partial success. Success may equate to a 50% osseous fill in 50% of the defects (Sepe et al., 1978). Root resorption may also occur with grafts (Dragoo and Sullivan, 1973). In some studies new attachment is seen only at the apical extent of defects near periodontal ligament and a long junctional epithelium extends below the alveolar crest of grafted sites (Moskow et al., 1979, Listgarten and Rosenberg, 1979).

Egelberg (1987) questions whether osseous grafts demonstrate the regeneration of new periodontal attachment. Conversely, Bowers et al. (1982)

reports osseous grafts promote regeneration of periodontal ligament, cementum and bone. Control of coronal inflammation limits root resorption and extended junctional epithelium (Bowers et al., 1982).

3. Guided tissue regeneration. Nyman et al. (1982) find guided tissue regeneration clinically effective in treating isolated osseous defects. The guided tissue regeneration technique uses membrane filters to isolate healing periodontal defects from gingival connective tissue and epithelium. Upon healing, bone and attached periodontal ligament reach the level of the bone surrounding the defect. Attachment forms coronal to the alveolar crest without concommitant bone formation (Lindhe et al., 1984, Gottlow et al., 1984, 1986).

The model of guided tissue regeneration describes healing in terms of granulation tissues arising from different sources (Figure 1B). Periodontal ligament granulation tissue regenerates periodontal ligament, cementum and supporting alveolar bone (Nyman, 1982a, 1982b, Isidor et al., 1986). Granulation tissue arising from bone causes root resorption and ankylosis of roots debrided of periodontal ligament and not protected by epithelium (Karring et al. 1984). Granulation tissue arising from gingival connective tissue does not provide new attachment and leads to root resorption (Nyman et al., 1980, Andreasen, 1981, Magnusson et al., 1985). Long epithelial attachment formed in the absence of guided tissue regeneration protects the tooth from resorption or ankylosis by bone or gingival connective tissue cells (Karring et al., 1980, 1984, Nyman et al., 1980).

Guided tissue regeneration is clinically effective in regenerating periodontal defects including molar furcation defects (Becker et al., 1987, Pontoriero et al., 1987, 1988). While these results are promising, guided tissue migration using membranes is best suited to isolated defects

Figure 1. Models of Wound Healing

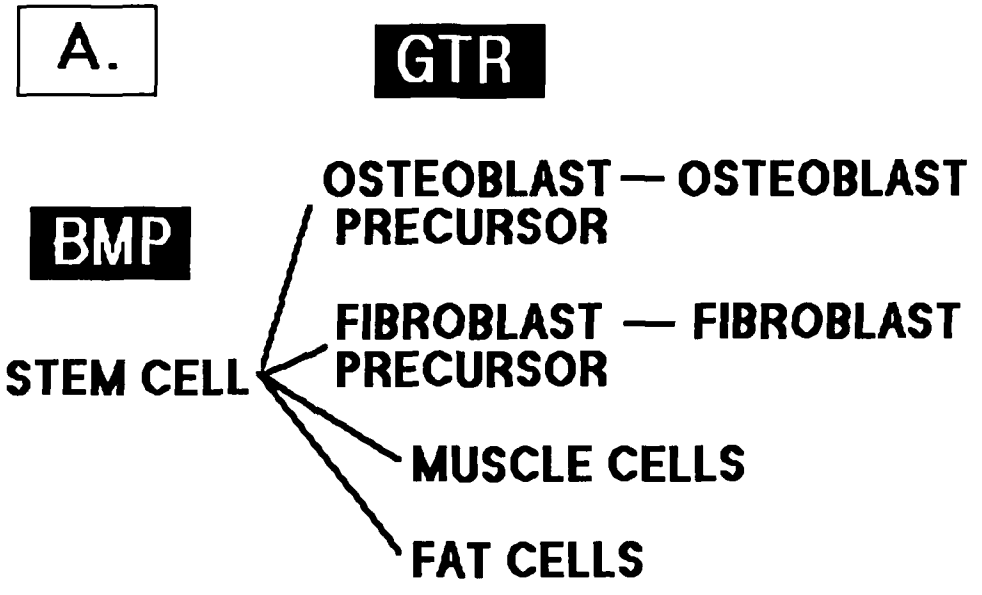
Figure 1A. Cellular Mechanisms

Mesenchymal stem cells, under the influence of bone morphogenic protein (BMP), aggregate, proliferate and differentiate into osteoblasts and form bone tissue. The theory of guided tissue regeneration (GTR) suggests proliferation of partially differentiated cells since adjacent connective tissues produce different healing responses. Both bone and periodontal ligament are regenerated from periodontal ligament granulation tissue suggesting both cell types may occur in the periodontal ligament.

Figure 1B. Guided Tissue Regeneration

The model of guided tissue regeneration suggests granulation healing tissue from gingiva causes root resorption and will not regenerate attachment. Granulation tissue from bone leads to both root resorption and ankylosis. Epithelial cells prevent attachment, but protect tooth from resorption. Only granulation tissue originating from the periodontal ligament regenerates the attachment apparatus.

A.



B.

Guided Tissue Regeneration

Tissue	Response
Gingiva	Root resorption, lack of regeneration
Bone	Ankylosis and Resorption
Epithelium	Protects root, lack of regeneration
PDL	Regeneration

(Egelberg, 1987) and bone regeneration is limited to vertical defects (Gottlow et al., 1986).

Regeneration appears possible with clinical techniques not excluding gingival connective tissues (Ellegaard et al., 1974, Becker et al., 1986) or bone (Bowers et al., 1982) which suggests the cellular model for guided tissue regeneration is overly restrictive and other modes of regeneration may be possible.

From a cellular perspective, guided tissue regeneration implies subpopulations osteoblast-like cells and fibroblast-like cells exist in the periodontal ligament since both bone and connective tissues regenerate from the periodontium (Figure 1A). Although granulation tissue originating from bone hypothetically causes bone resorption and ankylosis, membranes placed in guided tissue regeneration do not exclude bone cells from the healing defect. Gottlow et al. (1984, 1986) suggests that periodontal cells proliferate more rapidly than bone cells and does not rule out distinct cells types in the periodontal ligament and bone.

B. Rationale for Tissue Culture Studies.

Melcher (1976) notes a lack of experimental data supporting conceptual models of periodontal wound healing and suggests study of periodontal ligament cells in tissue culture as a model for wound healing. Culturing periodontal cells may enhance knowledge of periodontal healing mechanisms and improve the clinical success of regenerative procedures. Listgarten (1986) further suggests wound healing is significant to the progression of periodontitis since spontaneous healing follows episodes of tissue destruction.

Current observations of periodontal wound healing support the proliferation of partially differentiated cell populations and are more consistent

with the model of guided tissue regeneration than the model of bone morphogenic protein (Figure 1A). In vitro labeling experiments suggest periodontal cell populations proliferate from self renewing perivascular precursors. Differentiated cells continue to undergo clonal replication and migrate to adjacent periodontal ligament, bone and cementum under both physiologic conditions and in wound healing (Gould et al., 1980, McCulloch and Melcher, 1983a, 1983b, Davidson and McCulloch, 1986, Iglhaut et al., 1988).

Guided tissue regeneration is amenable to in vitro studies since distinct cell populations in the periodontal ligament are suggested and cellular transformation is not postulated. Hypothetically osteoblasts, fibroblasts, cementoblasts and precursors cells exist in the periodontal ligament and partake in regeneration. These cell populations should be demonstratable in vitro. Gingiva should have a distinct cell populations from periodontal ligament (Figure 1B). Bone-like cells may culture from the periodontal ligament, although differences between the bone and periodontal ligament cells seem likely (Figure 1B). Thus characterization of cells from periodontium provides a cellular basis for testing and interpreting the model of guided tissue regeneration and gives insight into healing mechanisms.

Periodontal tissues include a diversity of cell types including fibroblasts, myofibroblasts, cementoblasts, alveolar osteoblasts, endothelial cells, epithelial cells and defense cells characterized primarily by histology (Berkovitz and Shore, 1982). Two readily identifiable cell populations in periodontal explants are osteoblasts and fibroblasts.

C. The Osteoblast Phenotype.

Osteoblasts form a syncytium which joins with osteocytes and bone lining cells to maintain and renew bone. Osteoblasts form bone matrix. Osteocytes

may be osteoblasts incorporated in bone lacunae during osteogenesis. Osteocytes remain in contact with other bone cells via cellular processes within bone canaliculi. Bone lining cells may be unique cells of osteoblast lineage or resting osteoblasts; they line external and internal surfaces of bone matrix and might control internal bone environment. Bone cells are connected if tested electrically and appear to have either close or gap junctions joining adjacent cells. Bone has a composite microstructure of surprising strength and resilience that remodels in response to forces acting on the entire bone (Messer, 1982, Rodan and Rodan, 1984).

Osteoblasts both form organic bone matrix and mineralize the matrix. A similar osseous matrix is formed in membranous bone, in endochondral ossification, in Haversian systems (osteons), and in wound healing. Osteoblasts morphologically resemble other protein secreting cells: nuclei are displaced away from matrix and a prominent rough endoplasmic reticulum and Golgi apparatus are found between the nucleus and bone matrix. Organic matrix appears as osteoid subjacent to active osteoblasts and is later mineralized. Osteoblasts synthesize type I collagen which forms 90% of bone organic matrix. Osteoblasts also contain collagenase. A diversity of other proteins, glycoproteins, and phospholipids are formed and are thought to initiate and guide osteoid mineralization (Rodan and Rodan, 1984).

1. Bone collagen. Bone collagen is almost exclusively type I with small amounts of type V (1.5%). Type III collagen (found in some pathological conditions) may also be isolated. Type I collagen is a hybrid composed of two alpha(1) and one alpha(2) chains. Differences which may exist between bone collagen and soft tissue collagen fibrils occur as post translational events. Crosslinking in bone collagen utilizes the hydroxyallysine pathway whereas most soft tissues utilize the allysine pathways. Bone collagens tend to be

more hydroxylated. Both differences are age and tissue dependent. Hydrated demineralized bone has a larger pore size (1.53 nm) between collagen molecules than soft tissue type I collagen (1.33 to 1.53 nm) and differences may exist in the organization of fibrils between hard and soft tissues. Potential sites for mineralization exist within and between collagen fibrils. In the overlap zone collagen molecules are arranged in a quasi-hexagonal array and between these regions pores exist within collagen lattices. After fibril formation, molecular backbone movements allow variability in intermolecular spacings. Mineralization causes the matrix to become rigid. A number of crystallization sites and a variability of crystal size could potentially be accommodated by the matrix (Veis and Sabsay, 1987).

2. Non-collagenous bone proteins. Non-collagenous proteins synthesized by bone cells (most likely osteoblasts) include osteocalcin, osteonectin, bone phosphoproteins, bone sialoprotein, bone proteoglycan, bone morphogenic protein and bone proteolipid. Bone proteolipid is associated with osteoblast matrix vesicles and de novo calcification (Anderson, 1984). Organic matrix or osteoid does not appear to undergo a significant conformational change prior to mineralization suggesting collagen is not restructured prior to mineralization. Specific inhibitors may be removed from osteoid prior to mineralization or specific nucleating molecules may initiate mineralization. Non-collagenous proteins may orientate hydroxyapatite crystals, regulate their growth, or stabilize final crystal configurations. Thus, non-collagenous proteins of bone may play a significant role in initiating and regulating mineralization (Boskey and Posner, 1984).

a. Osteocalcin (bone gla protein). Osteocalcin is released by osteoblasts in response to 1,25-$(OH)_2$-vitamin D_3 (Lian et al., 1985) and is abundant in most species representing about 15% of the non-collagenous

proteins of bone, however, in man osteocalcin represents only 1-2% of the non-collagenous bone matrix. Osteocalcin is found only in bone and dentin and may provide a specific serum indicator of osteoblastic activity (serum levels are 5-10 ng/ml). Osteocalcin is also used to characterize osteoblasts in vitro. Osteocalcin is cleared from serum in minutes by glomerular filtration. The clinical value of osteocalcin may be as a diagnostic indicator of metabolic diseases in bone (Lian and Grundberg, 1988).

Human osteocalcin is a 49 residue (fully sequenced), 6,000 dalton protein. It contains 3 residues of the calcium binding amino acid gamma-carboxyglutamic acid (Gla) also found in prothrombin and factors VII, IX, and X. Gamma-carboxyglutamic acid forms by post translational, vitamin K and bicarbonate dependent, carboxylation of glutamic acid (Hauschka et al., 1982). The physiologic role of osteocalcin remains unclear since apparently normal bone synthesis occurs in warfarin treated animals, although vitamin K deficient rats show abnormal closure of epiphyses. Osteocalcin appears late in the mineralization of bone, after hydroxyapatite crystals (Price, 1985). The molar ratio of osteocalcin to bone collagen or mineral is fairly constant (Lian and Gundberg, 1988). Although osteocalcin binds Ca^{+2} weakly, it has a strong affinity for hydroxyapatite. Osteocalcin may regulate growth, orientate hydroxyapatite crystals, or stabilize crystal size (Boskey and Posner, 1984, Posner, 1985, 1987). Bone particles deficient in osteocalcin resorb at half the rate of normal bone particles in vitro (Lian and Grundberg, 1985). Osteocalcin is chemotactic for monocytes (Mundy and Poser, 1983).

b. Other Bone Proteins. Osteonectin is a 38,000 dalton phosphorylated glycoprotein that binds specifically to hydroxyapatite and covalently to collagen fibrils. Osteonectin causes calcification of type I collagen in vitro. Although osteonectin is found primarily in bone, other

cells in tissue culture including periodontal ligament, tendon, and skin cells release osteonectin (Triffitt, 1987). Osteonectin is also found in platelets (Stenner et al., 1986). Osteonectin is present at mineral formation sites and may bind hydroxyapatite crystals to collagen matrix or control hydroxyapatite crystal orientation and growth (Boskey and Posner, 1984).

Bone sialoprotein, which comprises 8-12% of non-collagenous bone matrix, was originally described in bovine bone as a 23,000 dalton molecule. Forty per cent of the molecule is carbohydrate and about half of the carbohydrate is sialic acid. Forty five per cent of the amino acids are aspartate and glutamate with 2-4 phosphates per molecule in this acidic glycoprotein (Fisher, 1983b). The cell receptor binding amino acid sequence of osteopontin (bone sialoprotein) is identical to fibronectin and vitronectin (Oldberg et al., 1986). Osteopontin binds tightly to hydroxyapatite and may act in mineralization and in cell attachment (Triffett, 1987).

Bone proteoglycans may control tissue hydration and nutrition, cell to cell interactions, and mechanical properties of bone (Boskey and Posner, 1984). Proteoglycans may affect collagen fibril and fiber formation (Triffitt, 1987). Bone proteoglycans are much smaller than the large hydrated proteoglycans of cartilage and have either one or two chondroitin sulfates (a glycosaminoglycan) attached to a short protein core (Fisher et al., 1983a).

3. Mineralization. Mechanisms guiding osteoid mineralization are incompletely understood. Matrix vesicles act as nucleation sites for de novo calcification by concentrating calcium and phosphorous to form crystals which eventually rupture the vesicles (Anderson 1984). Matrix vesicles may not be required to mineralize lamellar bone. Although the generic structure of bone type I collagen is not unique, post-translational modifications may provide attachment sites for specific nucleating matrix proteins, or alter the

collagen array to favor mineralization sites within intermolecular pores or fibrillar gaps. Molecules such as adenosine triphosphate (ATP), pyrophosphates and glycoproteins may inhibit nonspecific mineralization of collagen matrix or specific proteins may nucleate mineralization. Other non-collagenous proteins may control crystal size and coat bone crystals to reduce solubility (Kahn et al., 1984).

4. Alkaline phosphatase. Alkaline phosphatase occurs primarily as a membrane bound, high molecular weight, glycoprotein enzyme associated with osteoblasts and mineralization. Serum alkaline phosphatase levels increase during active bone formation. The stereo-specific inhibitor of alkaline phosphatase, levamisol, inhibits calcification in vitro. In hypophosphatasia mineralization of bone matrix is impaired and serum alkaline phosphatase levels are diminished (Kahn et al., 1984). Alkaline phosphatase may break down pyrophosphate, an inhibitor of mineralization or act as an adenosine triphosphate (ATP) activated Ca^{+2} transport protein and/or phosphorous transport protein (Messer, 1982). Alkaline phosphatase is a characteristic enzyme of osteoblasts, but isoenzymes exist in the kidney, liver, placenta, and intestine (Rodan and Rodan, 1984).

5. 1,25-$(OH)_2$-vitamin D_3 and osteoblasts. 1,25-$(OH)_2$-vitamin D_3 acts as a steroid hormone with a metabolic half life of 2-3 hours (Bell, 1985). It prevents rickets in children and osteomalacia in adults by maintaining normal serum levels of calcium and phosphorous. 1,25-$(OH)_2$-vitamin D_3 stimulates alkaline phosphatase activity and osteocalcin in osteoblasts and modulates bone cell differentiation (Norman et al., 1982, Nijweide et al., 1986).

D. Tissue Culture Studies of Periodontal Cells.

1. Gingival fibroblasts. Gingival fibroblast subtypes differ in

growth rate, collagen synthesis and complement binding. (Bordin et al., 1984, Barber et al., 1984, Hassel and Stanek, 1983).

Clusters of apatite-like crystals appear on extracellular fibrils after cultivating gingival fibroblasts for two months adjacent demineralized tooth wafers. Hypothetically, substances from tooth structure induce fibroblasts to form osteoid (Rose et al., 1981). Substances from extracts of bone, dentine and cementum increase collagen and total protein synthesis in gingival fibroblasts (Sommerman et al., 1984, 1987) .

Human gingival contains type I (91%), type III (9%), type IV (<1%), and type V (<1%) collagens (Narayanan and Page, 1983).

2. Periodontal ligament fibroblasts. Fibroblasts and epithelial cells culture from human periodontal ligament. Fibroblasts trysinize more easily than epithelial cells and form a dense multilayer outgrowth after trypsinization (Blomlof and Otteskog, 1981, Ragnarsson et al., 1985).

Human periodontal ligament cell lines have two morphologies when viewed with transmission electron microscopy (Rose et al., 1987). Comparable subtypes are seen in tissue sections (Yamasaki et al., 1986). Cells rich in glycogen and microfilaments with periodic dense nodes may be cementoblasts and a second population of cells without these features fibroblasts. Similar cell populations are observed in gingival cultures (Rose et al., 1987). Garant et al. (1982) describes microfilaments with periodic dense nodes connecting to extracellullar microfibrils at fibronexi in transeptal periodontal ligament fibroblasts from a squirrel monkey.

Receptors for epidermal growth factor are found on periodontal ligament fibroblasts, preosteoblasts and prechondrocytes suggesting osteoblasts precusors may be found in the periodontal ligament (Cho et al., 1988).

Rat periodontal ligament fibroblasts maintain type I and type III col-

lagen synthesis in tissue culture at levels similar to periodontal ligament type I collagen (94%), type III collagen (16-18%) and type V collagen (<1%) (Limeback and Sodek, 1979, Sodek and Limeback, 1979). Different clones of periodontal ligament produce different collagen ratios suggesting distinct populations of fibroblasts exist in the periodontal ligament (Limeback et al., 1983).

3. Osteoblast-like cells. Recent reports suggest cells from human periodontal ligament have osteoblast-like characteristics including high alkaline phosphatase levels and increased levels of cyclic adenosine monophosphate (c-AMP) in response to parathyroid hormone stimulation (Kawase et al., 1986, Piche et al., 1987, Somerman et al., 1988).

E. Cell Characterization.

In tissue culture osteoblast and fibroblast phenotypes differ in 1,25-$(OH)_2$-vitamin D_3 modulated (and basal) alkaline phosphatase activity (Luben et al., 1976, Majeska and Rodan, 1982, Beresford et al., 1986, Lomri et al., 1988), in osteocalcin release (Beresford et al., 1984, Skjodt et al., 1985) and in mineralization (Bellows et al., 1986, Ecarot-Charrier et al., 1983, 1988, Bhargava et al., 1988). Alkaline phosphatase is a characteristic of osteoblasts and possibly periodontal ligament cells (Kawase et al., 1986, Piche et al., 1987). Osteocalcin is an specific antigenic protein associated with osteoblasts. Osteoblasts release substantial quantities of osteocalcin in response to 1,25-$(OH)_2$-vitamin D_3. Mineralization is the functional phenotype of the osteoblasts, and recent studies support mineralization in tissue culture as a valid assay for osteoblasts. Fibroblasts do not produce alkaline phosphatase, osteocalcin or mineralize in culture and are readily distinguished from osteoblasts.

F. Objective

The objective of this study is to characterize, as osteoblast-like or fibroblast-like in tissue culture, cell populations from the attached gingiva, periodontal ligament, and debrided cementum. Hypothetically, both osteoblast and fibroblast phenotypes can be cultured from the periodontal ligament. Cell populations from attached gingiva and bone should be distinct. Characterization human periodontal cells will test the cellular model of guided tissue regeneration, contribute to knowledge of periodontal wound healing mechanisms, and facilitate future studies of periodontal wound healing.

II. METHODS

A. Cell Explants

1. Source of explants. Periodontal ligament and cementum derived cells were explanted from tissue scraped from impacted third molars (9 patients, average age 21 years) obtained from the Oral Surgery Clinic. Gingival cells were cultured from periodontal tissue, normally discarded, (6 patients, average age 48 years) obtained from the Periodontal Clinic. Bone explants were obtained from Orthopedic Surgery (2 patients aged 57 and 63 years). All samples were obtained at the University of Texas Health Science Center under an exempt Institutional Review Board protocol. Samples were limited to material required for tissue cultures.

2. Tissue culture procedures. Periodontal cells were explanted, transferred, cultured and frozen using techniques described by Freshney (1983) and Blomlof and Otteskog (1981). Bone tissues were explanted as described by Beresford et al. (1984). Specific modifications of these techniques are described below. Cells were observed and photographed with a Olympus phase contrast microscope.

a. Periodontal ligament. Samples received from surgery were placed in sterile 50 mL tubes (Corning) containing Dulbecco's Modified Eagle's Medium (Gibco), taken immediately to the laboratory, and processed in a sterile environment. Samples were washed five times in phosphate buffered saline (Sigma). Periodontal ligament tissue was dissected from teeth with scalpels and reduced to pieces less than 4 mm in length. Tissue was again washed three times in phosphate buffered saline. Samples were distributed in 3.5 cm wells on a 6 well plate (Corning) and incubated in growth media consisting of Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum

(Sigma) and 1% antibiotic solution (Sigma antibiotic solution: penicillin 10,000 units/ml, streptomycin 10,000 mg/ml and amphotericin B 25mg/ml). Cultures were incubated at 37° C in an atmosphere of 5% CO_2 and 95% air. Growth media was changed twice weekly until a substantial outgrowth of cells was seen. Cultures were then trypsinized with trypsin-EDTA (Sigma T 5775) using the Sigma protocol and transferred to a 75 cm^2 plate (Corning). Confluent 75 cm^2 plates were defined as the first cell passage.

b. Gingiva. To reduce contamination and to remove epithelium and periosteum a 1 mm to 2 mm tissue border was removed on all sides of gingival samples. The tissue core was washed, reduced to fragments of less than 4 mm on a side and cultured as described for periodontal ligament cultures.

A specific modification was utilized to select for osteoblast-like gingival cells. The initial explant outgrowth was trypsinized and plated on a 24 well plate. When these cells reached confluence, samples of cells from each well were histochemically stained for alkaline phosphatase (section B.2). Wells with 25% or more cells staining were pooled and cultured as osteoblast-like gingival cells. This technique was used to isolate osteoblast-like gingival cultures AG-2 and AG-5, both obtained from a subset of 4 wells from 24 well plates. Fibroblast-like AG-6 was cultured from the same initial gingival explant as AG-5 as a subset of 3 wells, negative for alkaline phosphatase, which were selected from a 24 well plate.

c. Cementum. Cementum derived cells used a modified explant technique. Once soft tissues had been thoroughly debrided from a tooth, surface shavings of the mid third root were removed using a sterile scalpel. Cementum/dentin shavings were placed in sterile 3.5 cm wells in a six well plate, washed three times with growth media and immobilized under sterile glass coverslips until a cellular outgrowth occurred.

d. Bone. Fibrous and fat tissue were dissected from cancellous bone and discarded. Large bone fragments were reduced with a scalpel or ronguer to small fragments, which were washed five times with phosphate buffered saline (Sigma) to remove non-adherent cells prior to culture.

e. Fibroblasts. Dermal fibroblasts in the tenth through twentieth passage were generously supplied by Dr. Dana Graves.

B. Cell Characterization.

Cells derived from periodontal ligament, cementum and gingiva were passaged and characterized as outlined in Figure 2. Bone explants and dermal fibroblasts served as control cultures. Cells were characterized as being osteoblast-like or fibroblast-like based on alkaline phosphatase activity, osteocalcin release and mineralization, with each assay modulated by 1,25-$(OH)_2$-vitamin D_3 (a gift from Hoffman LaRoche Co.).

1. Alkaline phosphatase biochemical assay. The assay is illustrated in Figure 3A. Unused perimeter wells were filled with sterile water which virtually eliminated variance between wells from edge effects. Cells were dispensed 10,000 cells per 0.1 ml per well (using a Coulter counter) on a 96 well plate and allowed to recover overnight in growth media. Cells were rinsed with 200 uL phosphate buffered saline (Sigma) and then stimulated 0 nM, 1 nM, 10 nM and 100 nM of 1,25-$(OH)_2$-vitamin D_3 for 48 hours in 200 uL of Dulbecco's Modified Eagle's Medium containing 0.1% serum bovine albumin (Sigma) and 1% antibiotics (Sigma). Assays were run in triplicate. Cells lysates were characterized for alkaline phosphatase activity as described by Stagni et al. (1979), but adapted to a 96 well plate. Alkaline phosphatase activity was assayed at pH 10 and 37° C in 150 uL 0.2 M diethanolamine (Sigma)

buffer containing 2 mM $MgCl_2$ and 5 mM paranitrophenol phosphate (Sigma) substrate. Reagents added to wells were rapidly mixed by pipetting to syncronize the start of the enzyme assay. Assays were quenched with 150 uL 1 N NaOH at 10 minutes. Phenol and bovine serum albumin (Sigma) protein standards were included with each assay and cellular protein content was assessed in separate wells using a BioRad protein assay (Baumgarten, 1985). Plates were read on a RPM 650 plate automatic plate reader (Dynatech) within one hour at 630 nm for protein and 410 nm for paranitrophenol.

2. Alkaline phosphatase histochemical assay. Cells were trypsinized, suspended in phosphate buffered saline, plated at approximately 10% confluence (diluted 1:10 from confluent cultures) and allowed to attach but not spread (approximately 1 hour) on glass coverslips in growth media. Cells were then fixed and histochemically stained for alkaline phosphatase (Sigma Kit No. 85).

3. Osteocalcin assay. The assay is illustrated in Figure 3B and is similar to the assays of Beresford et al. (1984) and Auf'mkolk et al. (1985). Cells were plated at confluence in 3.5 cm wells of 6 well plates (Corning) and cultured for 24 hours in growth media. Cells were stimulated in triplicate wells with test media containing 0 nM or 10 nM 1,25-$(OH)_2$-vitamin D_3 (plate 1), and 0 nM or 100 nM 1,25-$(OH)_2$-vitamin D_3 (plate 2) in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 1% antibiotics (Sigma), 1% newborn bovine serum, 2 mM glutamine, 50 ug/ml vitamin C, and 10 nM vitamin K (Sigma). After 24 hours supernatants were discarded and the test media replaced. After 48 hours cell supernatants and media controls were placed in labeled microcentrifuge tubes and frozen at -70° C until radioimmunoassayed for osteocalcin.

The experiment was repeated in a crossover protocol. Cells initially stimulated with 0 nM 1,25-$(OH)_2$-vitamin D_3 were stimulated with 10 nM or

100 nM 1,25-$(OH)_2$-vitamin D_3 in the second part of the assay, and cells initially stimulated with 10 nM or 100 nM 1,25-$(OH)_2$vitamin D_3 received a null stimulus in the second part of the assay. Initially, cells recovered in growth media containing 10% newborn bovine serum (Sigma) for 24 hours. Next cells were stimulated with 0 nM, 10 nM or 100 nM 1,25-$(OH)_2$-vitamin D_3 as described above for 24 hours and the media discarded and test media replaced. After 48 hours the media above the cells were collected in labeled microcentrifuge tubes and frozen until radioimmunoassayed for osteocalcin.

Osteocalcin concentrations of media were determined by radioimmunoassay (Gundberg et al. 1984). Osteocalcin standards, tracer and antibody were generously donated by Dr. Caren Gundberg of Yale University.

4. Mineralization assays. The mineralization assay was similar to that of Bellows et al. (1986). Confluent cells were cultured in 24 well plates using growth media (Dulbecco's modified Eagle's media supplemented with 10% newborn bovine serum and 1% antibiotics) supplemented with 50 ug/ml vitamin C, 10 nM vitamin K and 10 mM beta-glycerophosphate (Sigma). Test media included either growth media, basal mineralizing media or basal media supplemented with 100 nM dexamethasone or 100 nM 1,25-$(OH)_2$-vitamin D_3 in parallel cultures.

Mineralization was stained with von Kossa stain in triplicate wells (Putt, 1972). Linear patterns on over 50% of the plate were scored as a positive response, and staining on less than 10% of the plate was scored as a negative mineralization response. (Intermediate responses were noted.) Parallel culture were stained with alizarin red as a control (Putt, 1972).

5. Scanning electron microscopy. Mineralizing cultures from attached gingiva (AG-1) and periodontal ligament (PDL-4) were fixed in phosphate buffered 4% formaldehyde and 1% glutaraldehyde overnight. Specimens were then

Figure 2. Experimental Plan

Cells derived from explants of gingiva, periodontal ligament and cementum were characterized in tissue culture according to the plan shown. Cells were considered to be in the first passage when they reached confluence on a 75 cm^2 plate. Cells were cultured in sufficient numbers to accomplish characterization assays chosen to distinguish osteoblast and fibroblast phenotypes. High basal alkaline phosphatase activity modulated by 1,25-$(OH)_2$ -vitamin D_3 is a characteristic of osteoblasts. A very specific test for osteobasts is the release of osteocalcin in culture media in response to 1,25-$(OH)_2$-vitamin D_3. Mineralization characterizes the osteoblast phenotype. Cells lacking these attributes are fibroblast-like. Cell stocks were also preserved.

Experimental Plan

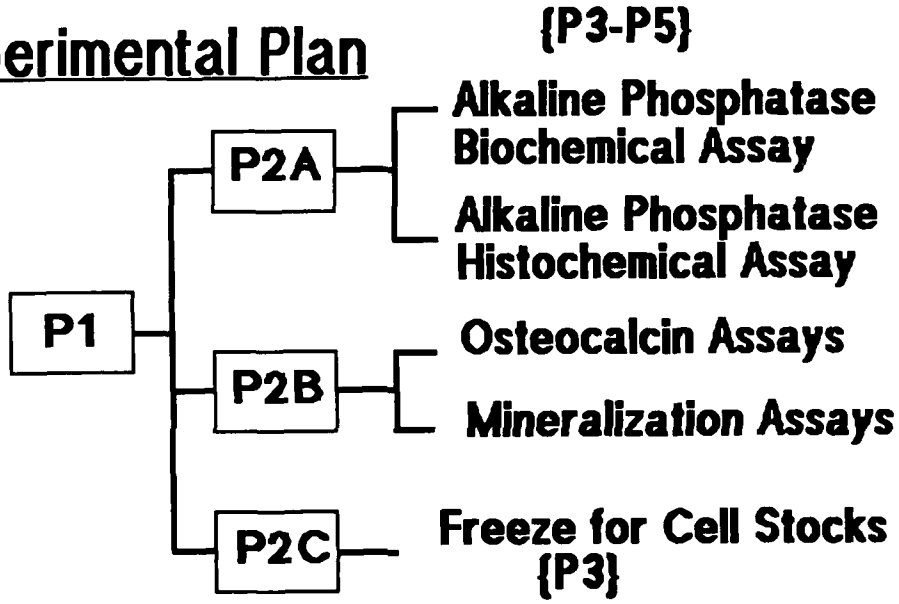


Figure 3. Characterization Assays

A. Alkaline Phosphatase Biochemical Assay

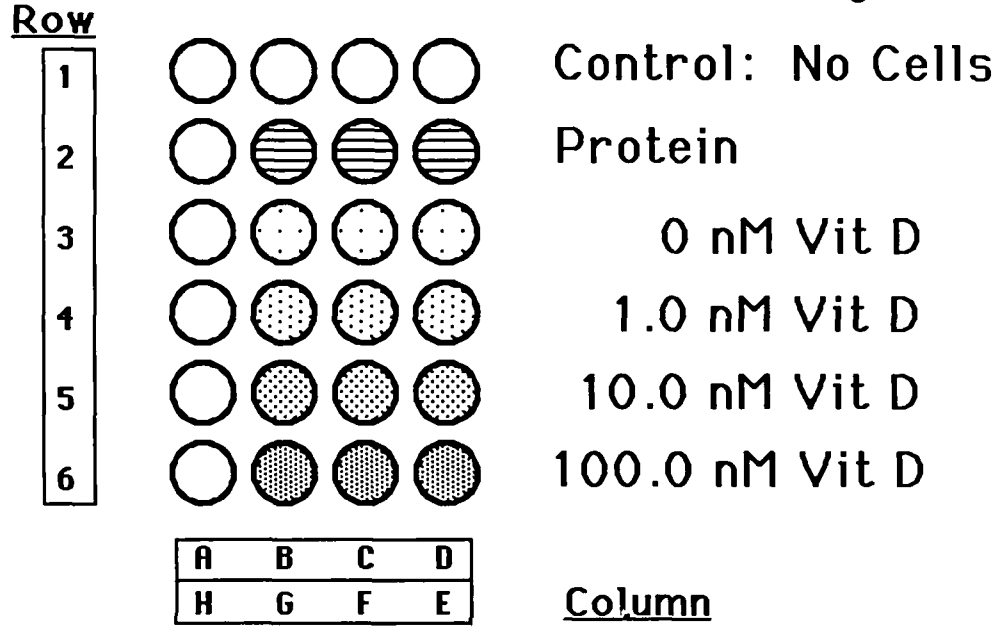
Cells were plated at 10,000 cells per well and cultivated 48 hours with 0 to 100 nanomolar (nM) 1,25-$(OH)_2$-vitamin D_3. Alkaline phosphatase activities of cell lysates were assessed. Results were expressed in nanomoles substrate reduced per microgram cellular protein per minute. Phenol and protein standards were included with each assay.

B. Osteocalcin Assay

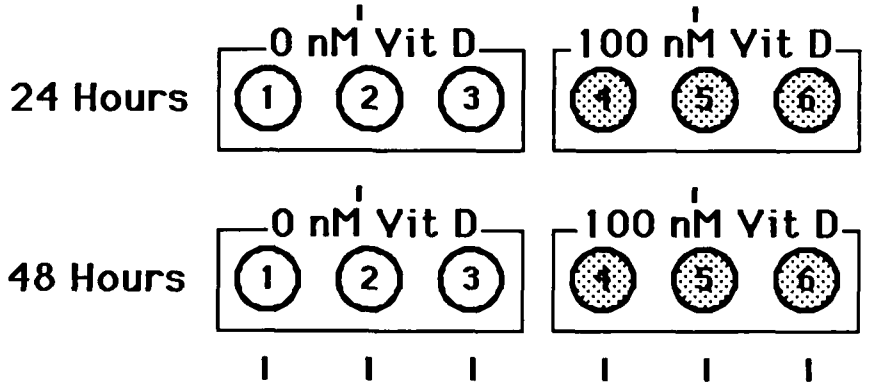
Media above the cells, which contained 0 nM or 100 nM (or 10 nM) 1,25-$(OH)_2$ -vitamin D_3, was collected after 48 hours of cell culture. Media were assayed for osteocalcin by radioimmunoassay. The osteocalcin assay was then repeated and the 1,25-$(OH)_2$-vitamin concentrations reversed: cells initially stimulated with 0 nM 1,25-$(OH)_2$-vitamin D_3 were stimulated with 100 nM.

A. Alkaline Phosphatase Assay

(1/4 96 Well Plate, Corning)



B. Osteocalcin Assay



rinsed overnight in 0.1 M phosphate buffered saline, post fixed in 1% Zetterqvist's buffered osmium tetroxide for 30 minutes, and rinsed twice in Zetterquist's buffer for 3 minutes. Specimens were dehydrated three times for 15 minutes in 70%, 95%, and 100% ethanol followed by five minutes in hexamethyldisilizane. Specimens were allowed to air dry in a dessicator, mounted and sputter coated three times with gold palladium before viewing.

6. Transmission electron microscopy. Mineralizing cultures from attached gingiva (AG-1) and periodontal ligament (PDL-4) were prepared as described for scanning electron microscopy through ethanol dehydration except they were fixed en bloc in saturated aqueous uranyl acetate for fifteen minutes prior to dehydration. After dehydration with 100% ethanol, specimens were rinsed twice for 10 minutes in propylene oxide. Specimens were then embedded in 1:1 propylene oxide:Epon 512 resin for 30 minutes, followed by 100% resin under 25 psi vacuum. They were embedded by the aligning a prelabeled, predried embedding mold filled with resin to the surface of the plate and cured in a 60° C oven for 18 to 20 hours. Sections were cut at approximately 90 angstroms and stained with saturated uranyl acetate for 3 minutes and Reynolds lead for five minutes. Pictures were taken on a JEOL 100CX electron microscope.

7. Immunohistochemisty. Two selected cultures from gingiva, one osteoblast-like (AG-5) and one fibroblast-like (AG-6) were immunohistochemically stained for keratin, factor VIII and S-100 (rabbit polyclonal antibodies from Dako), vimentin (rabbit monoclonal antibody from Dako), cytokeratin (mouse monoclonal antibody from Bectin-Dickinson) and Collagen IV (mouse monoclonal antibody from Cal. Biochem.) using supplemental reagents (from Vector). Parallel cultures were fixed in absolute ethanol for 15 minutes, dried and incubated with primary antibody (for example collagen IV binding

antibody) or phosphate buffered saline (controls) for twelve hours at 4° C and 100% humidity. After 3 two minute washes of 0.1 M phosphate buffered saline the slides were dried and incubated with the second labeling antibody (in this example biotin labeled horse antimouse antibody) for 30 minutes at 27° C in 100% humidity. Slides were rinsed three times in phosphate buffered saline, dried and incubated with amplifying reagent (avidin-biotin-peroxidase) for 30 minutes at 100% humidity at 27° C. Slides were rinsed 3 times with phosphate buffered saline, developed for 20 minutes using diaminobenzidine as a chromogen, rinsed with water, counterstained momentarily with alchohol free hematoxylin blue followed by 1% sodium bicarbonate, water rinsed and dried. Coverslips were mounted with 90% glycerol. Appropriate tissue sections (obtained from pathology) were run as controls. Positive cells stained brown and negative pale blue. Immunohistochemistry techniques were based on the avidin-biotin-peroxidase complex developed by Hsu et al. (1981) and have recently been reviewed by Taylor (1986).

C. Data Analysis.

Data was analysed using a Mann-Whitney U - Wilcoxon Sum W Test in a SAS statistical package using an alpha level of less than 0.01 for significance (unless noted). Biochemical alkaline phosphatase activity, alkaline phosphatase histochemical stain, and osteocalcin release were analysed statistically.

Mineralization and immunohistostaining data was treated as qualitative data and scored positive or negative according to criteria given.

III. RESULTS

A. Explant Cell Types.

The morphology of periodontal explants differed during initial cell outgrowth. After first passage all cell types assumed a similar fibroblast-like morphology in tissue culture.

Initial cultures of cells from periodontal ligament appeared fibroblast-like. In one explant culture, fibroblasts formed bands of cells between the base and side of the plate (Figure 4A) suggesting highly differentiated cells. Periodontal ligament cells were cultured without difficulty and reached confluence at one to three week intervals after each passage.

Cultures of cementum shavings appeared free of cells up to four weeks in culture. After four to six weeks an initial cellular outgrowth from root shavings was observed (Figure 4B). Four cementum derived cultures approached confluence on 75 cm^2 plates, but only two (CEM-1 and CEM-2) proliferated sufficiently in subsequent passage to accomplish an alkaline phosphatase assay. CEM-1 reached confluence at three to four week intervals and was fully characterized.

Gingival explants engendered a diverse outgrowth. The following patterns were observed within the first week in one or more explants: A rapid outgrowth of unattached cells cells, cells forming tubular structures, and sheets of cells with granular cytoplasm. Fibroblast-like cells proliferated rapidly and dominated the cultures at two to three weeks (Figure 4C). After the first passage cells appeared uniformly fibroblast-like in culture.

Gingival explants AG-5 and AG-6 were subcultured from the primary cell outgrowth of one tissue sample. Each appeared homogenous although AG-5 and AG-6 were not true clones. As characterized by immunohistological staining,

Figure 4. Periodontal Explants Observed with Phase Microscopy

A. Periodontal Ligament

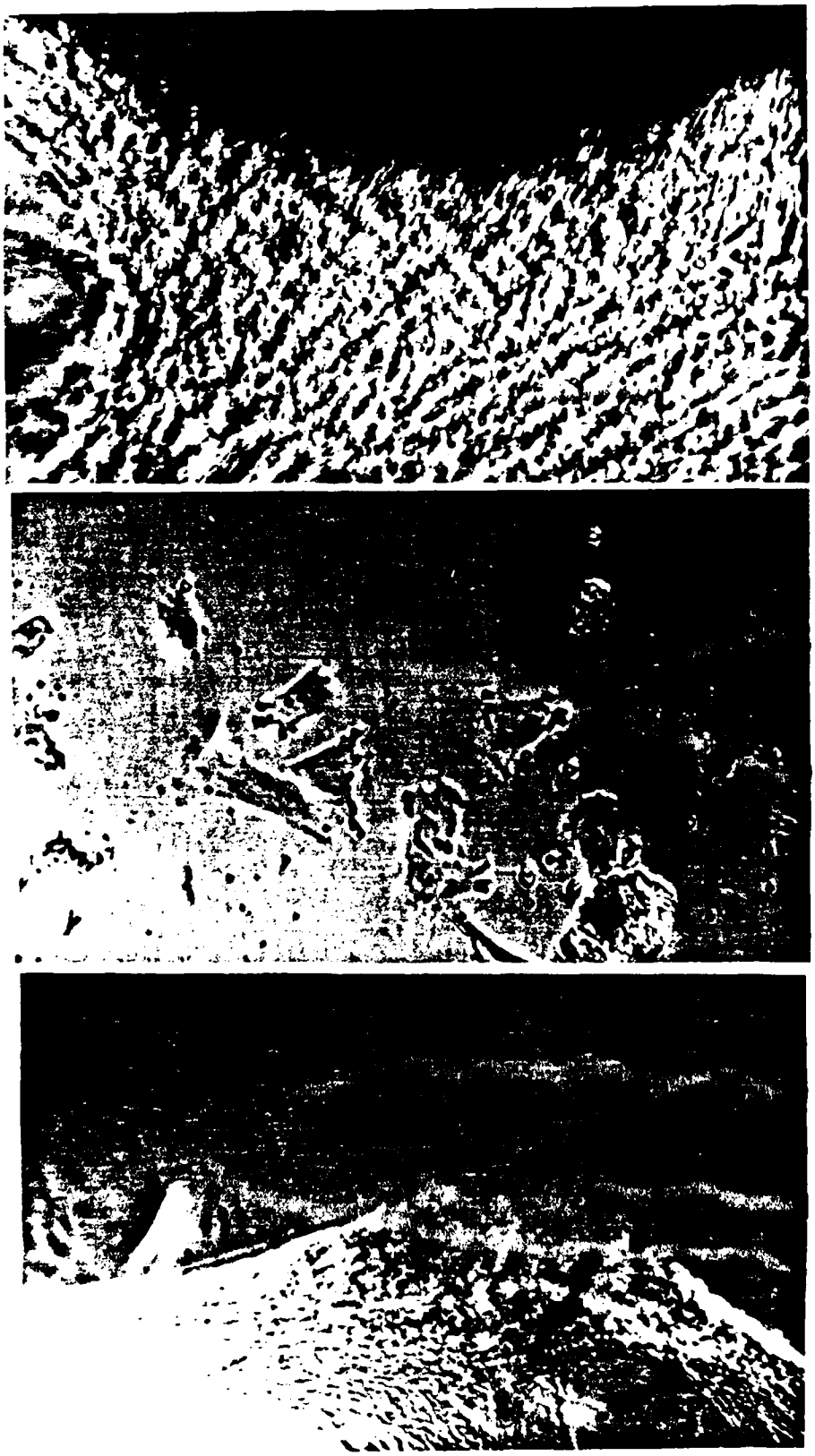
The initial outgrowth of cells from periodontal ligament explants appeared at 1 to 3 weeks. In this culture a well differentiated periodontal ligament explant formed cellular bands between the bottom and side of the tissue culture plate after 4 weeks in culture. Complex cellular arrangements did not persist once the cells were passaged (Scale = 140X).

B. Cementum

Cells were not observed at less than 4 weeks in cultures of debrided root shavings. In this explant cementum derived cells are seen in close proximity to cementum shavings after 5 weeks in culture. Dispersed dentin fragments appear as bright refractile granules near root shavings and on the surface of the plate (Scale = 700X).

C. Attached Gingiva

An outgrowth of fibroblast-like cells was observed from attached gingival explants in tissue culture at 1 to 2 weeks. In this example, an abundant, multilayered cellular outgrowth from attached gingiva is shown at 2 weeks (Scale = 350X).



both AG-5 and AG-6 subcultures stained negatively for keratin, cytokeratin, collagen IV, factor VIII, and S-100, indicating an absence of epithelial, endothelial and Langerhans cells. Both cultures stained positively for vimentin which is found in fibroblasts and osteocytes.

B. Alkaline Phosphatase Activity.

Control fibroblasts and osteoblasts showed marked differences in the biochemical alkaline phosphatase activity (Figure 5). Fibroblasts had levels of alkaline phosphatase activity which were less than 0.4 nM/ug-min and not modulated by 1,25-$(OH)_2$-vitamin D_3 and osteoblasts had levels above 0.8 nM/ug-min which increased in a dose dependent fashion after stimulation with 1,25-$(OH)_2$-vitamin D_3. A highly significant ($P<0.01$) difference in maximum alkaline phosphatase activity marked fibroblasts and osteoblasts.

Three populations of periodontal ligament cells (PDL-5, PDL-6, PDL-7) displayed less than 0.4 nM/ug-min alkaline phosphatase activity and were considered fibroblast-like. Three other populations of cells (PDL-1, PDL-3, PDL-4) displayed greater than 0.8 nM/ug-min alkaline phosphatase activity and were considered osteoblast-like (Figure 6).

Cementum derived cells were osteoblast-like in alkaline phosphatase activity with levels over 0.8 nM/ug-min when stimulated with 100 nM 1,25-$(OH)_2$-vitamin D_3, but basal alkaline phosphatase activities were well below 0.8 nM/ug-min (Figure 7).

Populations of attached gingival cells (AG-3, AG-4, AG-6) were fibroblast-like in having alkaline phosphatase levels below 0.4 nM/ug-min. Other gingival cultures (AG-1, AG-2, AG-5) were osteoblast-like in having 1,25-$(OH)_2$-vitamin D_3 simulated alkaline phosphatase activities above 0.8 nM/ug-min (Figure 8). Attached gingival cells AG-2 and AG-5 were subcultures

from primary explants which selected for alkaline phosphatase positive cells.

Alkaline phosphatase histochemical staining (Figure 9) was accomplished on a limited number of cultures and demonstrated a correlation between the percentage of cells staining for alkaline phosphatase histochemically and biochemical alkaline phosphatase activity of the cell lysates. Attached gingiva cells (AG-1) and periodontal ligament cells (PDL-1) had more biochemical alkaline phosphatase activity per percentage cells staining than control osteoblasts (BC-2) or cementum derived cells (CEM-1).

C. Osteocalcin.

Osteocalcin was not detected in the absence of 1,25-$(OH)_2$-vitamin D_3 stimulation. Cells stimulated with 1,25-$(OH)_2$-vitamin D_3 in the first cycle of the crossover osteocalcin assay stopped producing osteocalcin in the second cycle of the assay when 1,25-$(OH)_2$-vitamin D_3 stimulation ceased. Cells PDL-4 and AG-5 produced significant levels of osteocalcin when stimulated with 10 nM 1,25-$(OH)_2$-vitamin D_3. Cultures from attached gingiva (AG-1, AG-5) and from the periodontal ligament (PDL-1, PDL-4) formed significant levels ($P<0.01$) of osteocalcin when stimulated with 100 nM 1,25-$(OH)_2$-vitamin D_3 (Figure 10). A cementum derived cell (CEM-1) also showed significant ($P<0.05$) release of osteocalcin into the culture medium.

D. Mineralization.

Cell cultures from attached gingiva (AG-1, AG-5), periodontal ligament (PDL-1, PDL-4) and cementum (CEM-1) mineralized in culture when stimulated with either 100 nM 1,25-$(OH)_2$-vitamin D_3 or 100 nM dexamethasone in media containing beta-glycerophosphate. Mineralization was assessed using von Kossa stain (Figure 11). One cell (CEM-1) mineralized extensively in the absence of

Figure 5. Alkaline Phosphatase Activity of Control Cells

Dermal fibroblasts (FB-1 and FB-2) in the tenth through twentieth passage were used as negative controls and had less than 0.4 nM/ug-min of alkaline phosphatase activity. Bone explants (BC-1 and BC-2) were used as positive controls in the second through fifth passage and had greater than 0.8 nM/ug-min alkaline phosphatase activity. The alkaline phosphatase activity of bone explants was modulated by 1,25-$(OH)_2$-vitamin D_3.

The vertical axis is alkaline phosphatase activity in nanomoles of substrate paranitrophenol phosphate reduced per microgram of cell lysate protein per minute (nM/ug-min). The upper extent of each bar segment is the mean activity level (from 0) of the cell population. The horizontal axis is the cell type. Responses are given for stimulation with 0 to 100 nanomolar of 1,25-$(OH)_2$-vitamin D_3 for 48 hours.

Standard error of the mean ranged from 0.2% to 3%.

ALKALINE PHOSPHATASE ACTIVITY

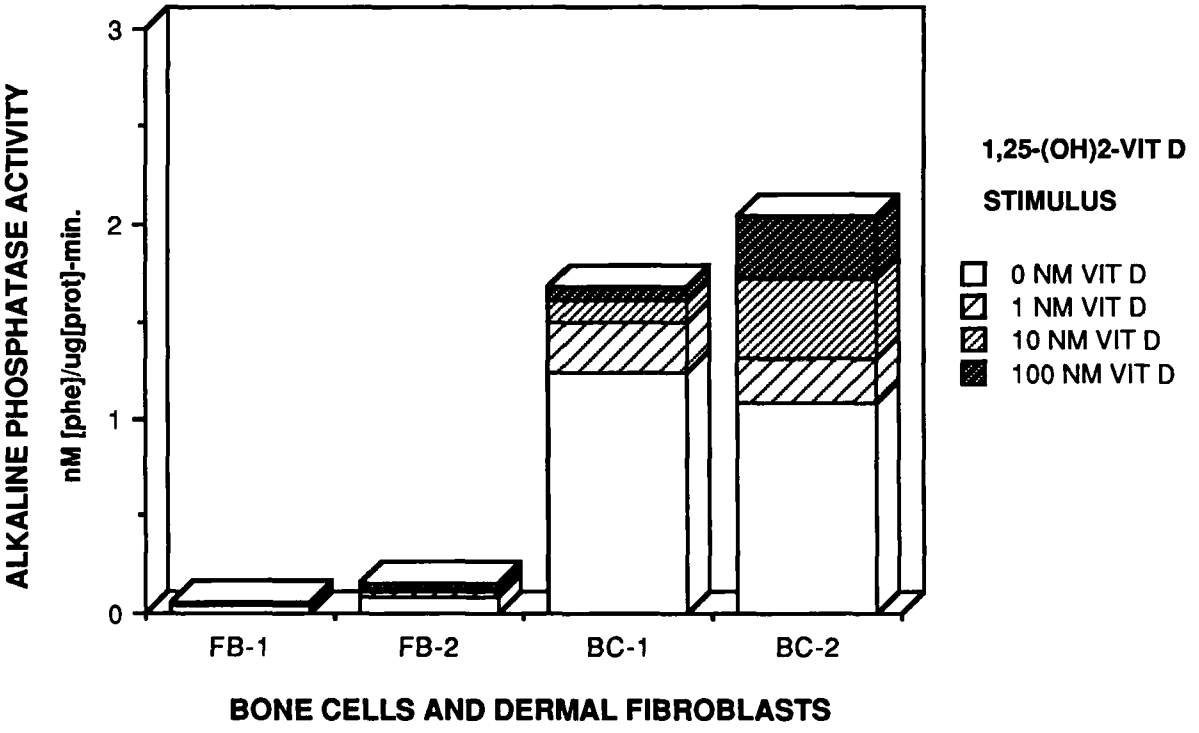


Figure 6. Alkaline Phosphatase Activity of Periodontal Ligament Cells

One group of cell populations cultured from the periodontal ligament (PDL-5, PDL-6, PDL-7) were fibroblast-like and had less than 0.4 nM/ug-min alkaline phosphatase activity. A second group of periodontal ligament cells (PDL-1, PDL-3 and PDL-4) were osteoblast-like and had greater than 0.8 nM/ug-min alkaline phosphatase activity with modulation by 1,25-$(OH)_2$-vitamin D_3. PDL-1, PDL-3 and PDL-4 also showed differences in 1,25-$(OH)_2$-vitamin D_3 modulation.

The vertical axis is alkaline phosphatase activity in nanomoles of the substrate paranitrophenol phosphate reduced per microgram of cell lysate protein per minute (nM/ug-min). The upper extent of each bar segment is the mean activity level (from 0) of the cell population. The horizontal axis is the cell type. Responses are given for stimulation with 0 to 100 nanomolar of 1,25-$(OH)_2$-vitamin D_3 for 48 hours.

Standard error of the mean ranged from 0.8% to 4%.

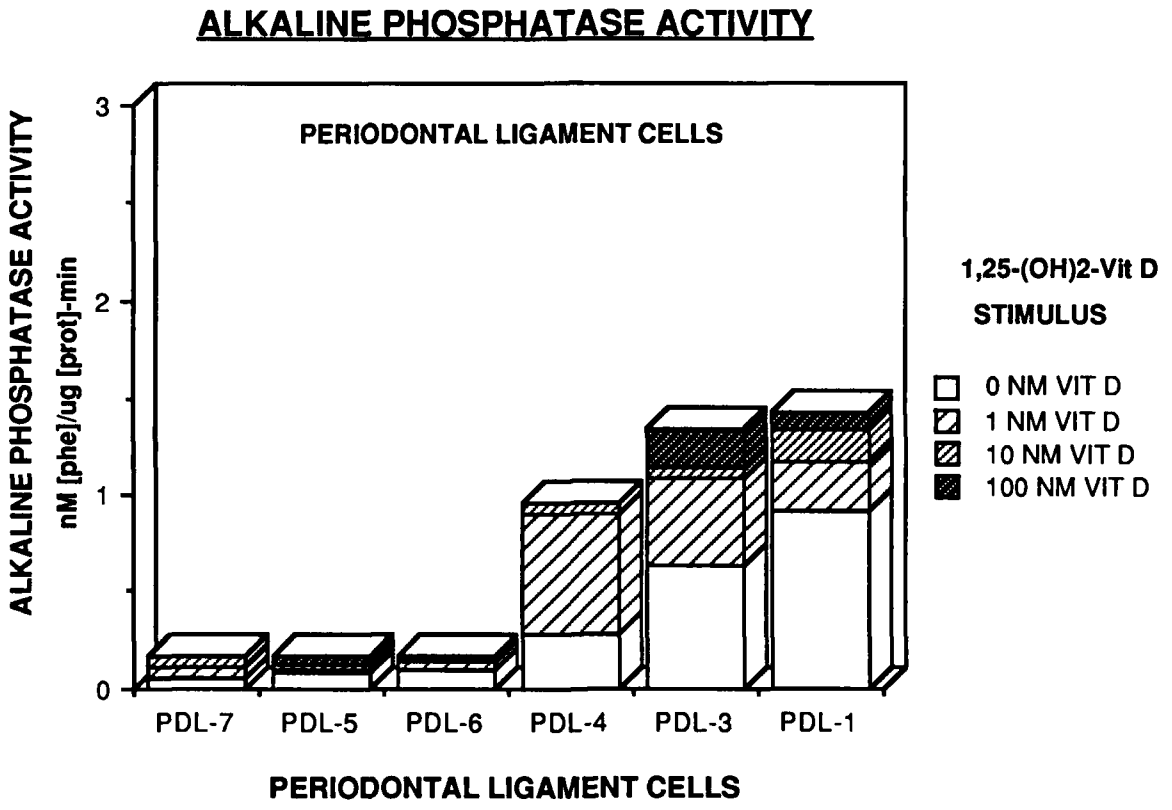

ALKALINE PHOSPHATASE ACTIVITY
PERIODONTAL LIGAMENT CELLS
ALKALINE PHOSPHATASE ACTIVITY
nM [phe]/ug [prot]-min
3
2
1
0
PDL-7
PDL-5
PDL-6
PDL-4
PDL-3
PDL-1
PERIODONTAL LIGAMENT CELLS
1,25-(OH)2-Vit D
STIMULUS
0 NM VIT D
1 NM VIT D
10 NM VIT D
100 NM VIT D

Figure 7. Alkaline Phosphatase Activity of Cementum Derived Cells

Cell populations derived from cementum (CEM-1, CEM-2) were osteoblast-like and had greater than 0.8 nM/ug-min alkaline phosphatase activity which was modulated by 1,25-$(OH)_2$-vitamin D_3. Cementum derived cells had lower basal alkaline phosphatase activities than bone cell controls.

The vertical axis is alkaline phosphatase activity in nanomoles of the substrate paranitrophenol phosphate reduced per microgram of cell lysate protein per minute (nM/ug-min). The upper extent of each bar segment is the mean activity level (from 0) of the cell population. The horizontal axis is the cell type. Responses are given for stimulation with 0 to 100 nanomolar of 1,25-$(OH)_2$-vitamin D_3 for 48 hours.

Standard error of the mean ranged from 0% to 4.5%.

ALKALINE PHOSPHATASE ACTIVITY

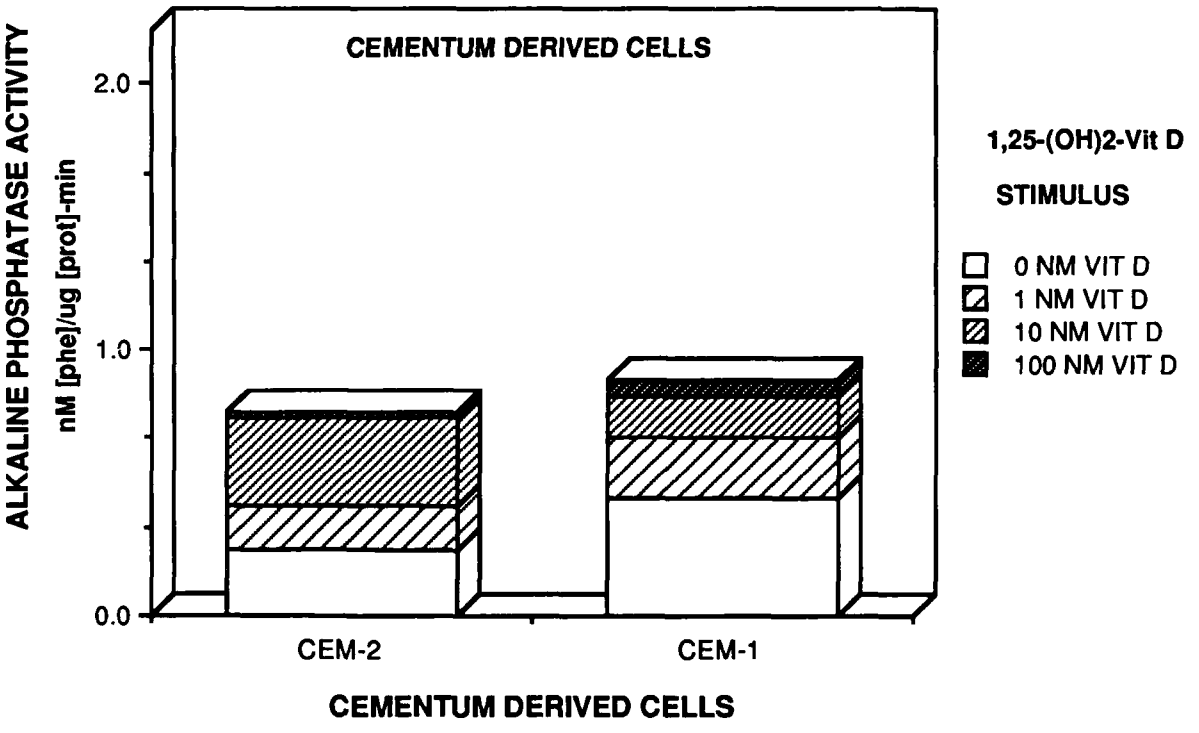


Figure 8. Alkaline Phosphatase Activity of Attached Gingival Cells

One group of cell populations cultured from attached gingiva (AG-3, AG-4, AG-6) were fibroblast-like and had less than 0.4 nM/ug-min alkaline phosphatase activity. A second group of cells (AG-1, AG-2 and AG-5) isolated from attached gingiva were osteoblast-like and had greater than 0.8 nM/ug-min alkaline phosphatase activity with modulation by 1,25-$(OH)_2$-vitamin D_3. AG-1, AG-2 and AG-5 also showed differences in 1,25-$(OH)_2$-vitamin D_3 modulation.

The vertical axis is alkaline phosphatase activity in nanomoles of the substrate paranitrophenol phosphate reduced per microgram of cell lysate protein per minute (nM/ug-min). The upper extent of each bar segment is the mean activity level (from 0) of the cell population. The horizontal axis is the cell type. Responses are given for stimulation with 0 to 100 nanomolar of 1,25-$(OH)_2$-vitamin D_3 for 48 hours.

Standard error of the mean ranged from 2% to 5% with the following exceptions: AG-2 at 1nM and 10 nM 1,25-$(OH)_2$-vitamin D_3 had 8% and 12% standard errors of the mean respectively.

ALKALINE PHOSPHATASE ACTIVITY

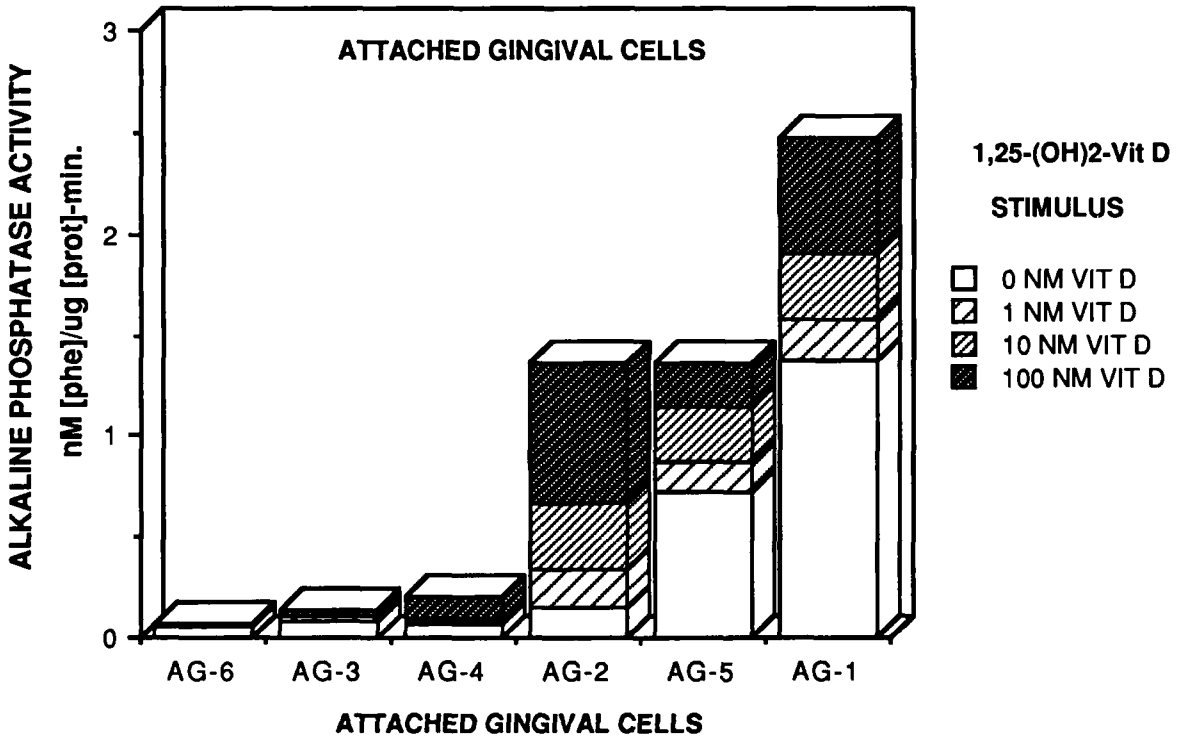


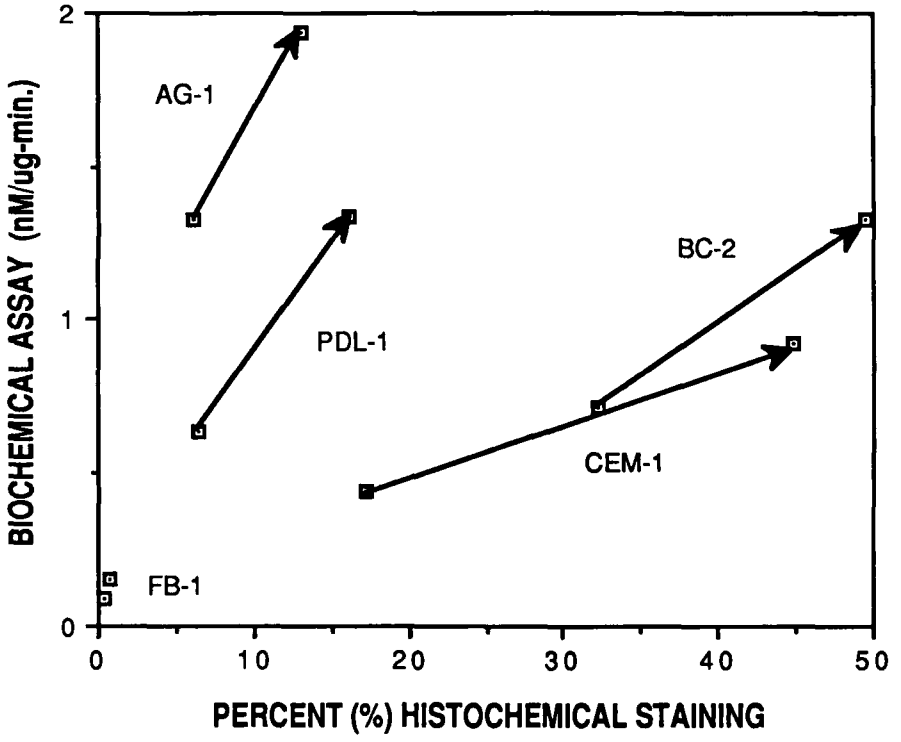
Figure 9. Correlation of Biochemical and Histochemical Alkaline Phosphatase Assays

Biochemical alkaline phosphatase activity in nanomoles/microgram-minute (nM/ug-min) was found to correlate with the percentage of cells staining histochemically for alkaline phosphatase. Biochemical alkaline phosphatase activity and percentage of cells staining for alkaline phosphatase increased proportionally in response to 100 nanomolar 1,25-$(OH)_2$-vitamin D_3 stimulation. In addition, attached gingival (AG-1) and periodontal ligament (PDL-1) cells had more biochemical alkaline phosphatase activity per percent cell staining than cells from bone (BC-2) or cementum (CEM-1). Dermal fibroblast (FB-1) had low alkaline phosphatase activity.

The vertical axis measures (biochemical) alkaline phosphatase activity in nanomoles of substrate paranitrophenol phosphate reduced per microgram of cell lysate protein per minute (nM/ug-min). The horizontal axis measures percentage of cells staining with alkaline phosphatase histochemical stain. Responses arrows are from the response after 48 hour stimulation with 0 nanomolar 1,25-$(OH)_2$-vitamin D_3 and to the response after 48 hour stimulation with 100 nanomolar 1,25-$(OH)_2$-vitamin D_3.

Standard errors of the mean ranged from 2% to 5%.

CORRELATION OF ALKALINE PHOSPHATASE ASSAYS



ALKALINE

PHOSPHATASE

RESPONSE ARROWS

FROM: 0 nM Vit D
TO: 100 nM Vit D

Figure 10 Osteocalcin Release in Response to
1,25-$(OH)_2$-Vitamin D_3 Stimulation

Cells from the periodontal ligament (PDL-1, PDL-4), cementum (CEM-1) and attached gingival (AG-1, AG-5) produced significant levels of osteocalcin in response to 100 nanomolar 1,25-$(OH)_2$-vitamin D_3. Another group of cells from the periodontal ligment (PDL-2, PDL-7) and attached gingiva (AG-2, AG-3, AG-6) did not release significant amounts of osteocalcin when stimulated with 100 nanomolar 1,25-$(OH)_2$-vitamin D_3. No osteocalcin release was detected in unstimulated cells.

Vertical axis represents osteocalcin release in nanograms of osteocalcin per milliliter supernatant. The horizontal axis is cell type. The horizontal line (at 3 nanograms osteocalcin) represents the detection limit of the assay.

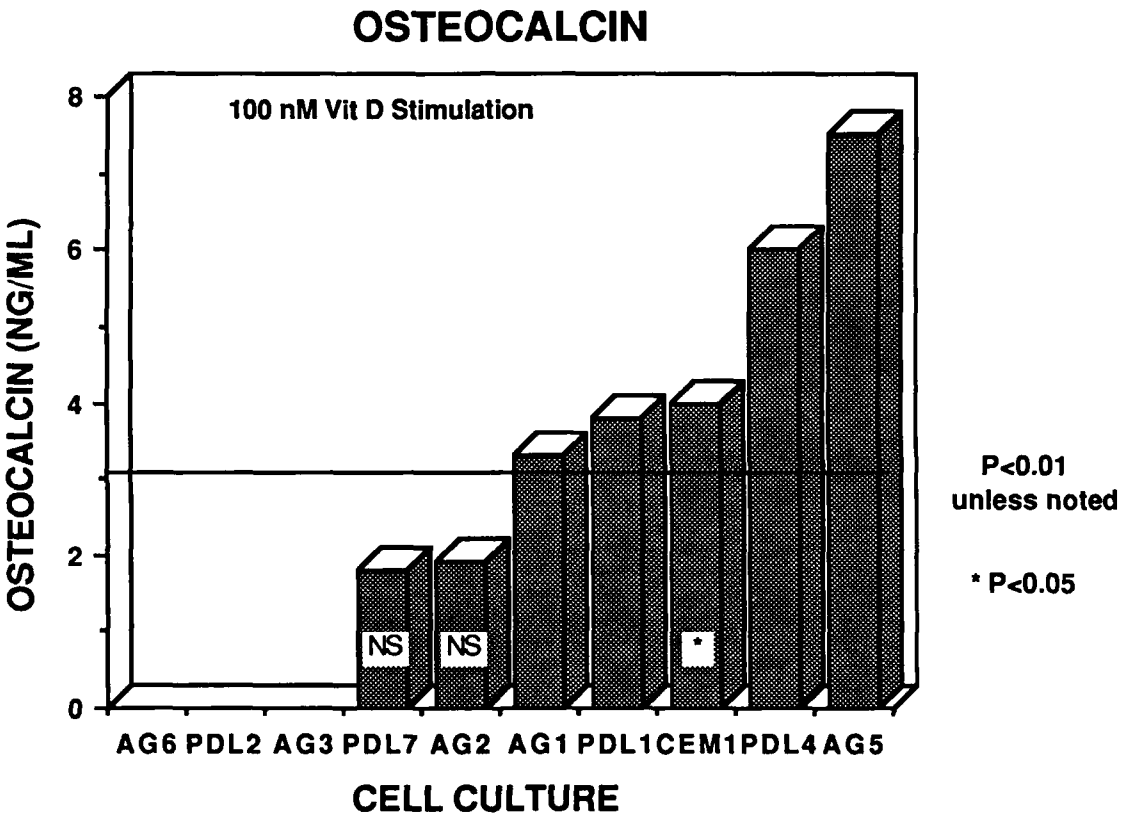

OSTEOCALCIN
100 nM Vit D Stimulation
OSTEOCALCIN (NG/ML)
8
6
4
2
0
NS
NS
*
AG6 PDL2 AG3 PDL7 AG2 AG1 PDL1 CEM1 PDL4 AG5
CELL CULTURE
P<0.01
unless noted
* P<0.05

Figure 11. Von Kossa Histochemical Stain.

A. The mineralizing attached gingiva cell population (AG-1) was grown in a media containing beta-glycerophosphate and stimulated with 1,25-$(OH)_2$-vitamin D_3. Staining is with von Kossa stain, which stains mineralized areas black, and with nuclear red counterstain, which stains cytoplasm pink and nuclei red. Black granular areas, areas of diffuse black patches, and linear black lines represent three patterns of mineralization seen. Scale = 200X.

B. Cell culture AG-1 shows the three mineralization patterns in greater detail with von Kossa stain and no counterstain. Mineralization exceeds the length of individual cells. Scale = 500X.

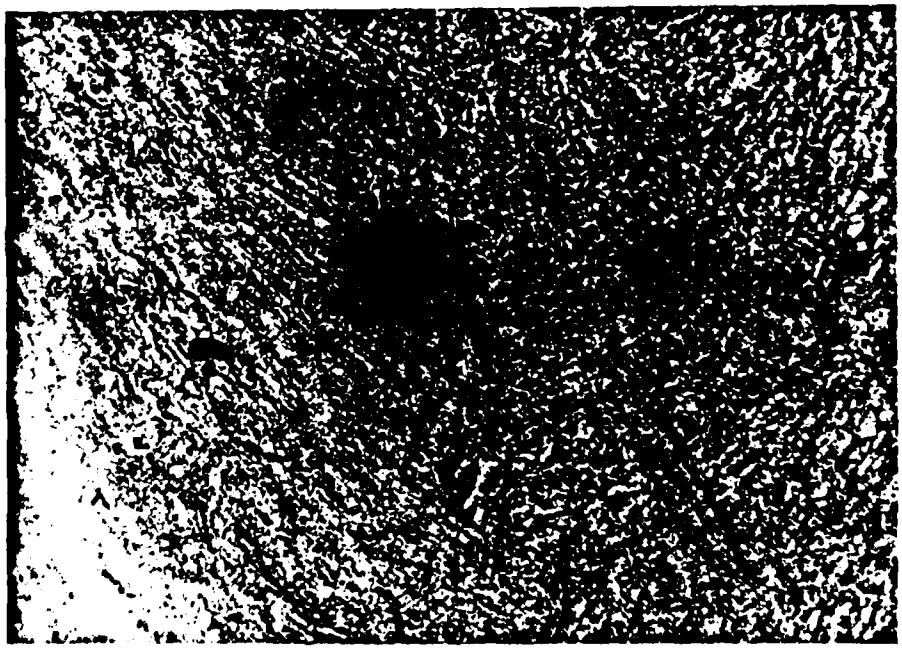


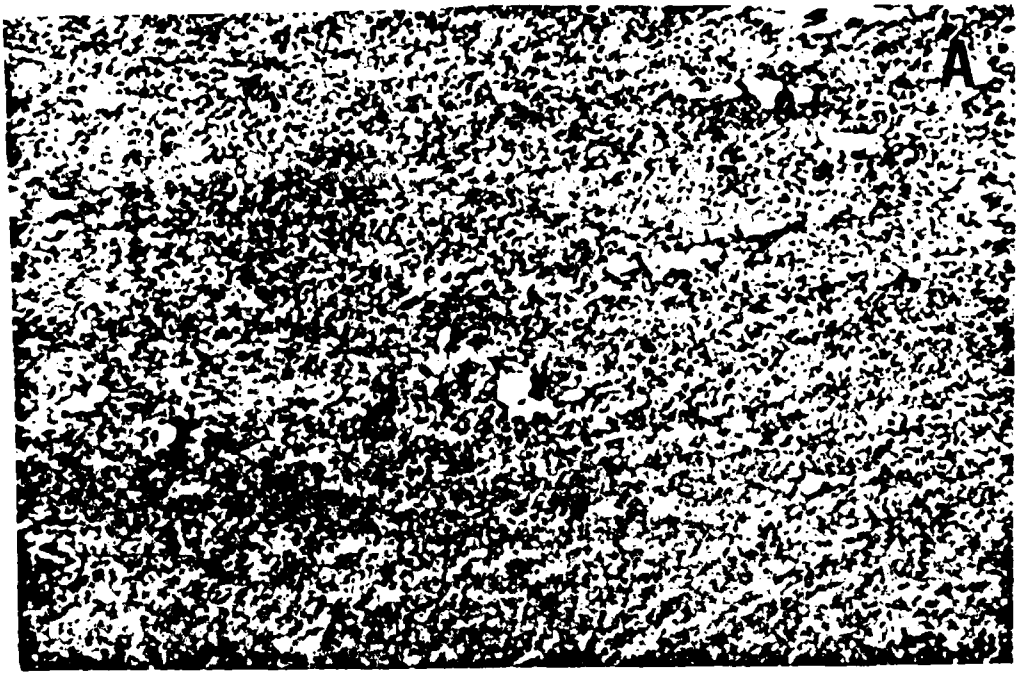
Figure 12. Scanning Electron Micrographs of Mineralizing Cells

A. Attached gingiva cells (AG-1) mineralized when stimulated with 100 nanomolar dexamethasone or 100 nanomolar 1,25-$(OH)_2$-vitamin D_3. Mineralization occurred underneath the cell layer and is not visible in this view. Artifactual separation of cells occurred with fixation. Scale = 20X.

B. At higher magnification boundaries between the attached gingival cells (AG-1) are indistinct. Fine cellular processes may be observed extending between adjacent cells. Scale = 2500X.

Figure 13. Scanning Electron Micrographs of the Mineral Layer

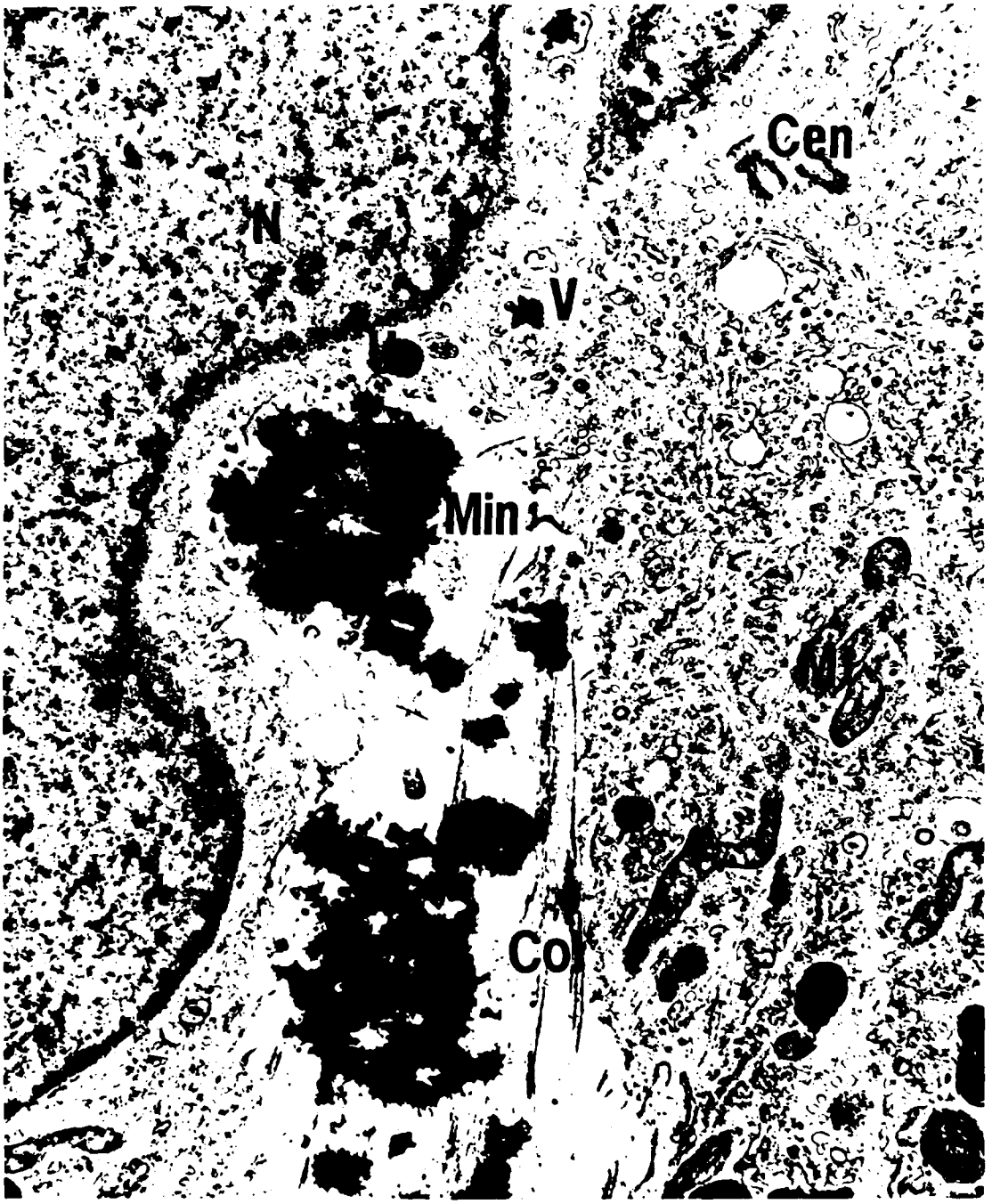
After 4 weeks of cell culture in media containing beta-glycerophosphate as an organic phosphate source, a mineral layer was seen adherent to the plate of the attached gingiva culture AG-1. More linear mineralization appeared in response to stimulation with 100 nanomolar 1,25-$(OH)_2$-vitamin D_3 (Figure B, Scale = 160X) than in response to stimulation with 100 nanomolar dexamethasone (Figure A, Scale = 160X).


A


B

Figure 14. Transmission Electron Microscopy of Mineralizing Cell Culture

This transmission electron micrograph of a mineralizing attached gingival (AG-1) cell was taken after 4 weeks in a culture stimulated with 100 nM 1,25-$(OH)_2$-vitamin D_3. The plane of the section is parallel to the plate and near the cell/mineral interface. Organelles including a nucleus (N), mitochondria (Mt) and centrioles (Cen) are clearly visible. A uniformly staining body (U) is also seen. The dark staining crystalline deposits external to the cell are mineral deposits (Min). These mineral deposits appear to form as round nodules which coalese. A small area of mineral surrounded by the cell appears to have matrix vesicles (V) or cytoplasmic extensions in close proximity to the mineral deposits. Partially mineralized collagen (Col) is seen external to the cell.


Cen
N
V
Min
Co

dexamethaxone or 1,25-$(OH)_2$-vitamin D_3 stimulation.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) provided a detailed view of mineralization by cell cultures from attached gingiva (AG-1) and periodontal ligament (PDL-4). Results for AG-1 were comprehensive and are presented in detail. SEM showed a confluent layer one or more cells thick with the suggestion of intercellular contacts (Figure 12). With the cell layer removed a mineral layer remained on the surface of the plate. SEM indicated that more extensive patterns of mineralization had formed beneath cells stimulated with 1,25-$(OH)_2$-vitamin D_3 than cells stimulated with dexamethasone (Figure 13). TEM revealed specific patterns of mineralization. Round nodules of mineralized crystal were observed which were in close proximity to cells. Partially mineralized collagen-like filaments were observed external to cells and in close proximity to mineral nodules (Figure 14). Mineralization was not observed with unstimulated AG-1 or PDL-4 cells.

E. Summary of Results

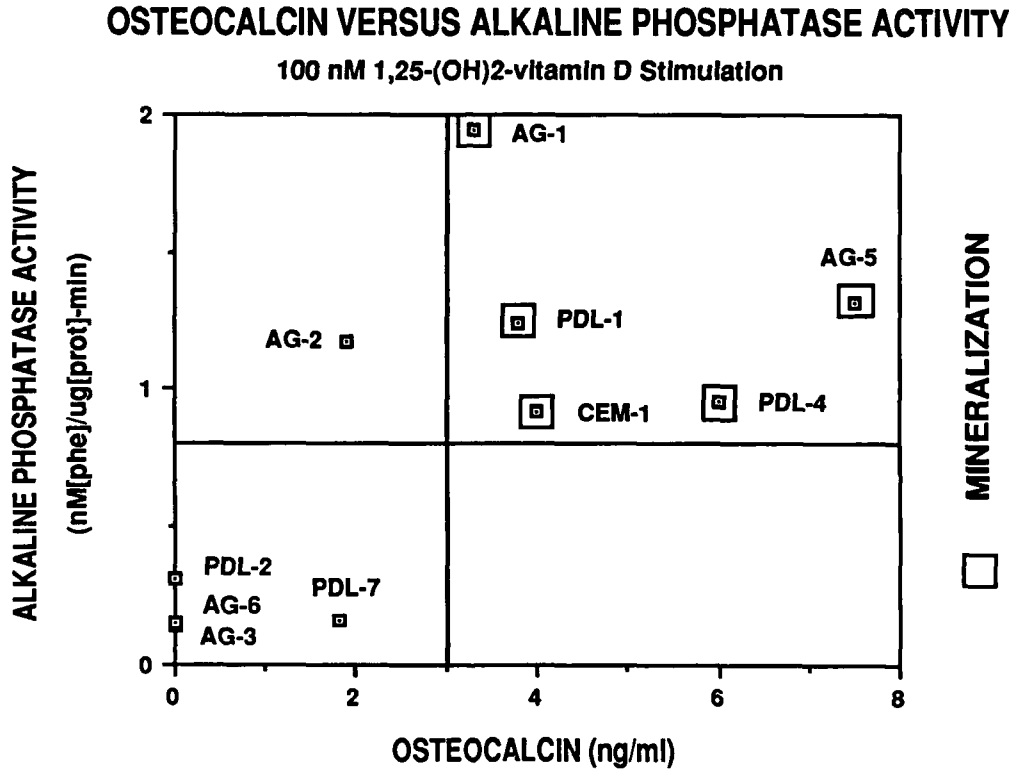
Cells isolated from both periodontal ligament (PDL-1, PDL-4), cementum (CEM-1), and attached gingiva (AG-1, AG-5) had significant levels of alkaline phosphatase activity, osteocalcin release, and mineralization in culture. Each effect was modulated by 1,25-$(OH)_2$-vitamin D_3. A second group of cells from periodontal ligament (PDL-2, PDL-7) and attached gingiva (AG-3, AG-6) had low alkaline phosphatase activity, no detectable osteocalcin release and did not mineralize in culture. Results are summarized graphically in Figure 15.

Figure 15. Mineralization Versus Alkaline Phosphatase Activity and Osteocalcin Release

Cells from periodontal ligament (PDL-1, PDL-4), cementum (CEM-1) and attached gingiva (AG-1, AG-5) had significant levels of alkaline phosphatase activity, osteocalcin release, and mineralization; these cell populations were considered osteoblast-like. A second group of cell populations were fibroblast-like (AG-3, AG-6, PDL-2, PDL-7) and had low levels of alkaline phosphatase activity, did not releasing significant levels of osteocalcin, and did not mineralize in culture. Cells with an intermediate response were also observed (AG-2).

The vertical axis is alkaline phosphatase activity in nanomoles of the substrate paranitrophenol phosphate reduced per microgram of cell lysate protein per minute (nM/ug-min). The horizontal axis represents osteocalcin release in nanograms of osteocalcin per milliliter (ng/ml) supernatant. Mineralizing cultures are indicated. Responses are given for stimulation with 100 nanomolar 1,25-$(OH)_2$-vitamin D_3 for 48 hours.

The horizontal line at 0.8 nM/ug-min represents significant osteoblast-like alkaline phosphatase activity ($P<0.01$) and the vertical line represents significant osteocalcin release ($P<0.01$ except for CEM-1 where $P<0.05$).


OSTEOCALCIN VERSUS ALKALINE PHOSPHATASE ACTIVITY
100 nM 1,25-(OH)2-vitamin D Stimulation
ALKALINE PHOSPHATASE ACTIVITY
(nM[phe]/ug[prot]-min)
OSTEOCALCIN (ng/ml)
MINERALIZATION
AG-1
AG-5
PDL-1
AG-2
CEM-1
PDL-4
PDL-2
PDL-7
AG-6
AG-3
0
1
2
0
2
4
6
8

IV. DISCUSSION

A. Periodontal Ligament Cells.

The combined assays of this study add significantly to prior evidence that both osteoblast-like and fibroblast-like cells may be cultured from periodontal ligament. Osteocalcin release modulated by 1,25-$(OH)_2$-vitamin D_3 is an extremely specific assay for osteoblasts (Beresford et al., 1984). Neither osteocalcin release nor mineralization had previously been demonstrated in periodontal ligament cell cultures. Cultures with high alkaline phosphatase activity, significant osteocalcin release, and mineralization, each modulated by 1,25-$(OH)_2$-vitamin D_3, provided strong evidence osteoblast-like cells may be cultured from the periodontal ligament. Fibroblast-like cells, lacking these responses, were also cultured.

Culturing osteoblast-like periodontal ligament cells confirms and extends the findings of Kawase et al. (1986). He characterized human periodontal ligament cell cultures which were similar to bone cell cultures in alkaline phosphatase activities and also characterized the periodontal ligament alkaline phosphatase enzyme as a bone-like alkaline phosphatase.

Multiple cell populations in periodontal ligament are consistent with the theory of guided tissue regeneration. Nyman et al. (1982a, 1982b) proposed a guided tissue regeneration model which suggested that the periodontal attachment apparatus, including cementum and collagen fibers, may regenerate from cells of periodontal ligament. The different subpopulations of periodontal ligament cells demonstrated in this study could potentiate healing in the various tissue compartments of the attachment apparatus.

B. Cementum Derived Cells.

Finding osteoblast-like characteristics in cementum derived cells is consistent with the findings of Melcher et al. (1986) who reported that cells from bone synthesized cementum-like tissue when cultivated on demineralized root slices. However, Melcher et al. (1986) also reported that cells from gingiva and periodontal ligament did not express an osteoblast-like phenotype, conflicting with this study.

Cementoblasts may be osteoblast-like but not osteoblasts. Lindskog et al. (1987a, 1987b) studied reimplanted teeth with scanning electron microscopy and suggested cementoblasts did not respond to parathyroid hormone and were a cell type distinct from bone cells. Rose et al. (1987) and Yamasaki et al. (1986) suggested human cementoblasts were a subpopulation of periodontal ligament cells containing branching bands of microfilaments with semiperiodic dense nodes and massive amounts of glycogen.

An easily repeated protocol developed as a part of this study allows the culture of cementum derived cells from root shavings and provides an opportunity for the further study of these presumed cementoblasts. Cementum derived cells grew from root chips after four to six weeks, proliferated slowly in culture, and displayed an osteoblast-like phenotype in terms of alkaline phosphatase activity, osteocalcin release, and mineralization. Isolating cementum derived cells in tissue culture may make possible development of an antibody specific to cementoblasts. Such an antibody would be of value in following cementum formation in periodontal wound healing studies and in determining the source of fibro-osseous lesions in oral pathology. Further studies of cementum derived cells might also clarify differences between the osteoblast and cementoblast phenotypes.

C. Attached Gingival Cells.

The guided tissue regeneration theory of Nyman et al. (1982a, 1982b) suggests gingival granulation tissue causes tooth resorption, which does not necessarily conflict with this study. In light of current evidence that many mediators of bone resorption act via the osteoblast (Chambers, 1985), osteoblast-like cells in the gingiva might modulate the resorption of root surfaces. Gingival inflammation releases factors such as prostaglandins and osteoclast activating factor, which has identity with interleukin-1 (Dewhirst et al., 1985; Gowen et al. 1983, 1986), and these factors may act on osteoblasts to indirectly activate osteoclasts (Thomson et al., 1986, 1987). Cells capable of resorbing mineralized matrix, whether monocytes, giant cells, or osteoclasts, appear to derive from hematopoietic mononuclear cell precursors (Mundy, 1987) which are clearly not specific to gingiva. The resorption of normal healthy bone requires osteoclasts (Chambers, 1985). Although little is known of dentin and cementum resorption, root resorption may be regulated in a manner similar to bone resorption. Lindskog (1988) notes morphological similarities between osteoclasts and dentinoclasts. Hypothetically, osteoblast-like cells in the gingiva could activate osteoclast-like cells on the root surface under certain environmental conditions. Resorption is rarely seen in the coronal aspect of the root. More commonly periapical root resorption occurs in association with a necrotic pulp and periapical granuloma (Shafer et al., 1983).

Alternatively, root resorption may be similar to the direct monocytic bone resorption seen in rheumatoid arthritis (Key et al., 1987) and not require gingival cells. These observations provide reasonable doubt that gingival connective tissue cells directly initiate root resorption, but do not rule out the possibility of an indirect influence.

Similar osteoblast-like cells were isolated from both periodontal ligament and gingiva suggesting cell populations of these tissues overlap. The ratios of cell types in periodontal tissues was not addressed in this study. Since selective techniques appeared necessary to consistently isolate osteoblast-like cells from the attached gingiva, quantitative differences may exist in cell populations common to both gingiva and periodontal ligament. Differences in cell populations may also exist which were not revealed in this study.

Finding osteoblast-like cells in cultures from gingiva as well as from periodontal ligament reflects the related and intermeshed anatomy and function of these tissues (Figure 16A). Both attached gingiva and periodontal ligament include collagen fibers which insert into cementum and bone (Ten Cate, 1985). The gingival, or supracrestal, fibers groups (dentogingival, alveologingival, circular and dentoperiosteal) are all in close proximity to the alveolar crest fiber group of the periodontal ligament (Ten Cate, 1985). Gingival fibers intermesh with periodontal fibers so that no clear anatomical boundary exists between the two tissues. Both gingiva and periodontal ligament have abundant oxytalan fibers which are not present in adjacent alveolar mucosa or bone (Soames and Davies, 1975, 1978) and both tissues lack a distinct periosteum. These observations support the finding of overlapping cell populations.

Healing studies suggest gingiva and periodontal ligament contain similar cells. Gingiva reportedly forms around teeth if alveolar mucosa is removed and healing occurs from the periodontal ligament (Lundberg and Wennstrom, 1987). Granulation tissue from gingiva and periodontal ligament both form tissue histologically similar to gingiva (Karring et al., 1975).

The boundary between attached gingiva and periodontal ligament is even

less clearly defined in the presence of bone loss. Tissues considered part of the periodontal ligament in health may be redefined as attached gingiva once bone loss has occurs and the boundary between periodontal ligament and attached gingiva apparently shifts apically (Figure 16B). Even if gingival and periodontal ligament cell populations are initially distinct, periodontal ligament cells might not completely "evacuate" tissues redefined by alveolar bone loss. Since the gingival biopsies of this study were from older patients with periodontal disease, some osteoblast-like gingival cultures may have been cultured from a portion of gingiva once anatomically part of the periodontal ligament. The presence of osteoblast-like cells in gingiva has clear implications towards wound healing regardless of their origin.

A mixed cell population in the periodontal ligament has the potential to replace the various tissue components of the attachment apparatus (Nyman et al., 1982a, 1982b) and some degree of regeneration may be possible from a similar mixed cell population in the gingiva. McHugh et al. (1988) describes coronal attachment to submerged roots distant (4-8mm) from the periodontal ligament, a healing response which appears to originate with gingival cells. Gantes et al. (1988) and Martin et al. (1988) report clinical success in regaining bone in furcations with a 67% average bone fill using coronally positioned flaps and making no attempt to exclude gingival tissues. These findings suggest additional clinical techniques might be developed to utilize cells from attached gingiva in regenerative wound healing.

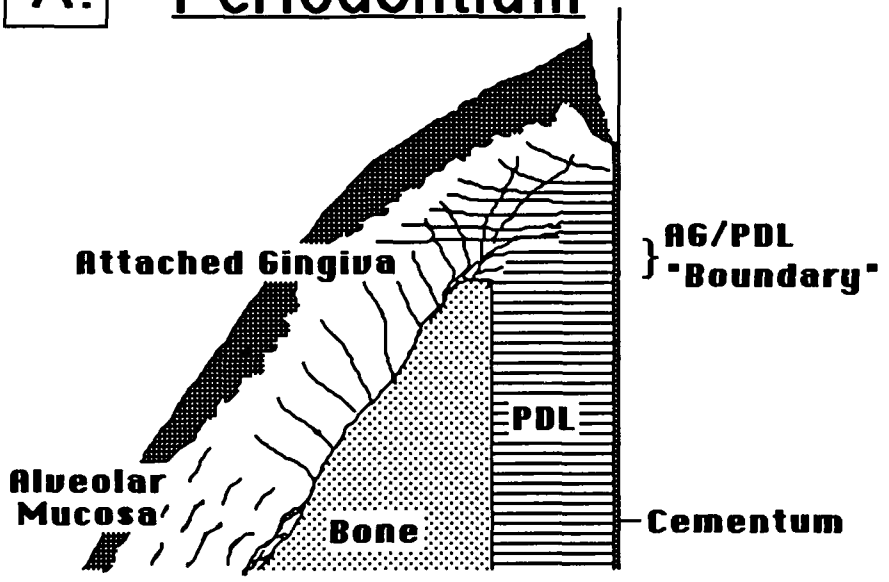
Finding osteoblast-like cells in gingiva has interesting implications for oral pathology. The peripheral ossifying fibroma is a common lesion of the gingiva, which often appears to originate from the interdental papilla and contains calcification in the form of trabeculae of bone, globules resembling acellular cementum, or diffuse granular calcification (Shafer et al., 1983).

Figure 16. Schematic of the Periodontium in Health and with Mild Bone Loss.

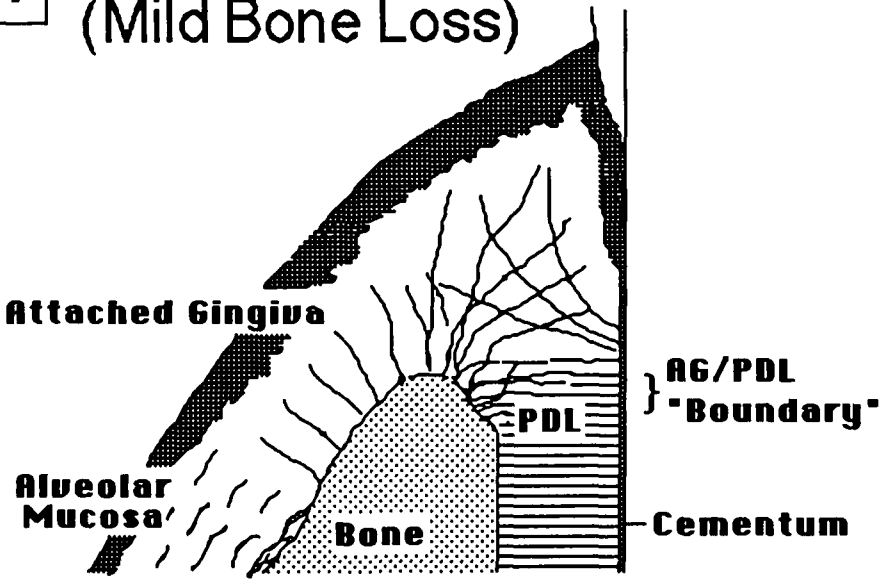
A. Periodontium. The periodontal ligament (PDL) and attached gingiva share similarities of form and function. Fibers of both the periodontal ligament and attached gingiva attach to bone and cementum. Neither the periodontal ligament nor the attached gingiva have a distinct periosteum and both contain oxytalan fibers. No distinct anatomical boundary exist between attached gingiva and periodontal ligament.

B. Periodontium with mild bone loss. Once bone loss has occurred the boundary between periodontal ligament and attached gingiva apparently shifts apically and the interface between periodontal ligament and attached gingiva becomes even more uncertain.

A. Periodontium



B. Periodontium
(Mild Bone Loss)



Findings of this study suggest that peripheral ossifying fibromas might represent the proliferation of cellular elements normally present in gingiva or periodontal ligament.

Hassal and Stanek (1983) demonstrate different gingival fibroblast subtypes and suggest selective proliferation of one subtype in response to a drug might induce gingival hypertrophy. Osteoblast-like and fibroblast-like cells in the attached gingiva affirm the presence of distinct subtypes. Osteoblast-like gingival cells might relate to gingival hypertrophy.

Rose et al. (1987) suggests glycogen rich gingival cell cultures reflect in vivo cementoblastic gingival cells similar to cementoblasts they describe in the periodontal ligament.

D. Mixed Cell Populations in Tissue Culture.

Mixed cell populations were used instead of clones to overcome difficulties of cultivating cells from the periodontium and to obtain general characterization data not possible with clones. Currently, very little is known of the characteristics of periodontal cells in tissue culture and few studies have characterized multiple periodontal cell cultures.

Certain responses in the characterization assays may be directly attributed to mixed cell populations. In some assays fibroblast-like periodontal cells demonstrated a tendency towards a osteoblast-like response, most likely due to the presence of osteoblast-like cells in these cultures, e.g. PDL-7 in Figures 6 and 10, AG-4 in Figure 8, and AG-2 in Figure 15. Gingival cell AG-2 presented a response intermediate between an osteoblast-like and fibroblast-like phenotype. AG-2 had a significant osteoblast-like response in alkaline phosphatase activity, but did not release significant amounts of osteocalcin. Low basal alkaline phosphatase activity in AG-2, which increased

significantly in response to 1,25-$(OH)_2$-vitamin D_3, appeared to be a characteristic of a mixed culture. Non-specific binding of substrate to a second cell type would explain the low basal alkaline phosphatase activity of the mixed cultures. The alkaline phosphatase activity after 1,25-$(OH)_2$-vitamin D stimulation would not be affected by background binding and would increase with stimulation as in AG-2. Mixed cultures also would have a lower basal alkaline phosphatase activity since the activity is expressed in terms of the total cell protein, which includes the protein of non-responding cells in a mixed culture. Increased biochemical alkaline phosphatase activity per percentage of cells staining (Figure 9) in cells from the gingiva and the periodontal ligament may have also resulted from mixed cultures.

Osteocalcin levels were 4 to 8 ng/ml (Figure 10) in osteoblast-like cell cultures from the periodontal ligament and gingiva and which compares to 5-10 ng/ml of serum (Lian and Gundberg, 1988). Levels over 10 ng/ml were reported in human bone cell cultures (Beresford et al., 1984). A variation in response is to be expected between different osteoblast cultures, but lower osteocalcin release may reflect mixed cultures since subcultured AG-5 showed the strongest response, 7.5 ng/ml.

Gingival cultures AG-5 and AG-6 were osteoblast-like, and fibroblast-like, respectively, selected from the same initial outgrowth of a single gingival explant. However, most cultures were not selected and displayed an osteoblast or fibroblast-like phenotype early in culture and remained stable through up to five passages as the cells were characterized. The tendency of a culture to display a predominant phenotype may relate to the release of self-regulating hormones.

Possibly certain results occurred due to mixed cultures which would not occur in clones. Cultures selected as subcultures based on alkaline

phosphatase histostain (AG-5, AG-6) were osteoblast-like and fibroblast-like. Also, immunohistochemistry on AG-5 and AG-6 showed no evidence of other cell types. However, only assays of clones will completely resolve this issue.

Specific growth factors or culture charateristics problably did not induce the appearance of osteoblast-like cells in an initially fibroblast-like population. Urist (1965) established that mesenchymal cells from tissues unrelated to bone, such as smooth muscle, were induced to differentiate into osteoblast precursors with bone morphogenic protein. Register (1973) hypothesized that citric acid could expose bone morphogenic protein within dentin and induce reattachment by gingival cells. The differentiation of osteoblast-like cells due to a bone inductive factor in culture is unlikely since control cultures were unaltered, not all cultures contained osteoblast-like cells, and dentin was not included in gingiva or periodontal ligament cultures cultures.

Some selection of cell types in tissue culture is likely. The cultivation method used appeared to select against epithelial cells as indicated by the immunohistochemical results. Selection of osteoblast-like cells from the attached gingiva was accomplished to prevent the overgrowth of these cells by gingival fibroblasts.

E. Mineralization Assay.

Periodontal cell cultures characterized as osteoblast-like in osteocalcin release and in alkaline phosphatase activity were found to mineralize in an osteoblast-like manner. Fibroblast-like cultures did not mineralize. The morphologic pattern of mineralization revealed small mineralized nodules in close proximity to cellular processes (Figure 14) resembling in vivo intramembranous mineralization as described by Bernard and Pease (1969) in fe-

tal rat calvaria. Koshihara et al. (1987) found cells cultured from femur periosteum of young male volunteers were osteoblast-like in 1,25-$(OH)_2$-vitamin D_3 modulated osteocalcin release and alkaline phosphatase activity. These osteoblast-like cell populations mineralized in tissue culture when stimulated with 10 nanomoles of 1,25-$(OH)_2$-vitamin D_3 in a pattern similar to that shown in Figure 11. Gerstenfeld et al. (1987) described mineral deposits in clusters (with linear patterns) and diffuse grey areas, similar to those shown in Figure 11, in cultures of chicken osteoblasts. The chicken osteoblast cultures also demonstrated co-deposition of collagen, osteocalcin and hydroxyapatite mineral. Thus periodontal cells in this study were osteoblast-like in their mineralization pattern when compared to both in vivo (Bernard and Pease, 1969) and in vitro (Koshihara et al., 1987; Gerstenfeld et al., 1987) studies of mineralizing bone cells.

V. SUMMARY

Cells populations from periodontal ligament, debrided cementum and attached gingiva demonstrated high alkaline phosphatase activity, significant osteocalcin release, and mineralization in culture, with each effect modulated by 1,25- $(OH)_2$-vitamin D_3. These highly specific assays suggest osteoblast-like cells were cultured. Other cells from periodontal ligament and attached gingiva lacked osteoblast-like traits and were characterized as fibroblast-like.

The model of guided tissue regeneration proposed by Nyman et al. (1982) is supported since distinct cell populations, potentially capable of regenerating the distinct components of the attachment apparatus, were found in the periodontal ligament. However, finding osteoblast-like cells in the both periodontal ligament and attached gingiva suggests that cellular healing mechanisms may be more complex than previously described.

A protocol developed for culturing cementum derived cells provides the opportunity to further characterize these cells. Cementum derived cells are of interest in studies of oral pathology and periodontal wound healing.

Osteoblast-like cells were isolated from attached gingiva as well as from periodontal ligament, although the isolation techniques used suggest osteoblast-like cells may be less numerous in gingiva. The presence of osteoblast-like cells in attached gingiva emphasizes anatomical similarities between attached gingiva and periodontal ligament and suggests regeneration of the attachment apparatus may be feasible from cells in attached gingiva.

Cells from the periodontium appear to provide an excellent *in vitro* model for further studies in periodontics including mineralization, pathology, pharmacology and healing mechanisms.

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